Vascular cell activation: mechanisms and consequences in human disease

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Dedicated to

Sean and Mary Diamond.

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Summary

Vascular cell activation: mechanisms and consequences in human disease.

At sites of injury/inflammation local vascular cell activation occurs. This results in the elaboration of cytokines, chemokines and, ultimately, the infiltration of interstitial tissues by peripheral blood leukocytes. Recent advances in the understanding of the paradigm of leukocyte trafficking and, in particular, the role of adhesion molecules, chemokines and chemokine receptors in this process are reviewed. Vascular cell activation, while essential in host defence, has pathological consequences in a variety of human diseases. The immunopathology of these processes in the development of atherosclerosis, vasculitis, thrombosis, vasoconstriction and allograft rejection is discussed in detail. We develop the concept that diverse stimuli perturb normal vascular homeostatic mechanisms and initiate the cascade of inflammatory events which leads to the ultimate phenotypic expression of these various diseases.

Growing evidence links systemic hypertension with the development of atherosclerosis, and glomerular hypertension with glomerulosclerosis. Evidence has been provided that mechanical strain acting on the vessel/glomerulus may be a trigger for antigen-independent vascular cell activation in these diseases. In the current study in vitro experiments were designed to focus specifically on the putative modulation of immune mediator expression by mechanical strain. Results demonstrate that mechanical strain can be either a proinflammatory or prosclerotic stimulus depending on the cell type used. In ECV-304 cells cyclic mechanical strain results in increased expression of chemokines (IL-8 and MCP-1), chemokine

receptor (CXCR4) and adhesion molecule expression (ICAM-1). In this study, increased expression of COX-2 in ECV-304 cells was also demonstrated in response to mechanical strain. The immunomodulatory effects of COX-2 following vascular injury and in animal models of inflammation have been the focus of much recent interest and the implications of such strain-induced increases in COX-2 expression, given the available evidence to date, are discussed. In contrast, exposure of mesangial cells to cyclic mechanical strain results in increased expression of the prosclerotic mediators TGF-β and CTGF, supporting a role for this stimulus in the ECM accumulation characteristic of glomerulosclerosis. No such increases occurred in mesangial cell expression of cytokine, chemokine or chemokine receptors in response to mechanical strain.

Finally, observations in both acute and chronic vascular disease suggest the existence of endogenous regulatory systems that act to restore homeostatic vascular tone, thrombogenicity and adhesiveness for leukocytes to normal. In this dynamic process a large number of anti-inflammatory molecules are elaborated. Experiments focused on the actions of lipoxins, a group of rapidly-acting anti-inflammatory eicosanoids - specifically the signal transduction events triggered by the activation of lipoxin receptors in endothelial and mesangial cells. The suppressors of cytokine signalling (SOCS) proteins are a recently identified family of proteins which inhibit cytokine-activation of the Janus kinase/signal transducers and activators of transcription (JAK/STAT) cascade. The structural features of these proteins, their mechanisms of action and possible physiological roles are reviewed. The role of the SOCS proteins in inflammatory-based disease states is to date unknown. Experiments were therefore undertaken to elucidate the role of SOCS in autoimmune inflammation using a rat

crescentic nephritis model. Increases in SOCS-3 and CIS-1 were demonstrated which appeared to correlate with disease activity. In addition, the profile of SOCS expression was examined in a model of alloantigen-driven inflammation - experimental cardiac transplant rejection. SOCS-3 expression was confined to the rejecting cardiac allografts and occurred throughout the time course studied whereas increases in SOCS-1 and CIS-1 expression, although present, were less sustained. The expression of a representative array of immune mediators implicated in the transplant immune response (cytokines, chemokines, chemokine receptors, adhesion molecules and COX-2) was also profiled in this model. The demonstration of SOCS expression in both these models implicates these endogenous inhibitors in the modulation of cytokine bioactivities in inflammation and host defence.

Aims

1. To assess the effect of cyclic strain on ECV-304 cell immune mediator (cytokine, chemokine and adhesion molecule) expression.
2. To examine the potential activation of glomerular endothelial and mesangial cells by cyclic mechanical strain.
3. To characterise the signal transduction events triggered by ligand-dependent activation of endothelial lipoxin and leukotriene receptors; specifically to assess putative MAP kinase activation.
4. To profile suppressors of cytokine signalling (SOCS) expression in glomerular endothelial and mesangial cells *in vitro*.
5. To assess renal SOCS expression in an experimental nephrotoxic nephritis model.
6. To profile the expression of SOCS in experimental acute cardiac transplant rejection and to investigate the temporal expression of cytokines and leukocyte trafficking determinants in this model.

Chapter 1.

Regulation of vascular cell activation in health.

Chapter 1

Regulation of vascular cell activation in health

1.1. Overview of endothelial cell, vascular smooth muscle cell and pericyte physiology in health

1.1.1. Endothelial cells

The vascular endothelium, located at the interface between blood and the vascular wall, is ideally positioned to modulate not only the activity of circulating blood components, but also the structure and function of the vessel wall. In health, the endothelium maintains vascular tone, provides a non-thrombogenic surface, is anti-adhesive for leukocytes and is a permeability barrier across which there is bidirectional exchange of nutrients and metabolites between blood and tissue extracellular fluid. In addition, endothelial cells synthesise the basement membrane collagens and proteoglycans upon which they rest, and, by the elaboration of growth factors and cytokines, mediate important growth-regulatory and immuno-modulatory effects (1). The endothelial surface is constantly exposed to humoral factors, inflammatory mediators and haemodynamic forces and thus, by virtue of its strategic position, is ideally placed to serve both as a sensor of these various signals and as an effector of the biological responses to them.

Endothelial cells and structural integrity

The endothelial cells of normal arteries form a continuous single layer of flattened cells, orientated with the long axis in the direction of blood flow. In areas of turbulent flow or altered shear, the cells are altered in shape. In high shear, for

example, they are elongated and, in low shear, cobblestone in shape (2, 3). The cell shape is maintained by the endothelial cell cytoskeleton consisting of actin microfilaments and microtubules. Cell-cell contact between endothelial cells is maintained by unique calcium-dependent adhesion molecules of the cadherin family in adherens junctions (4). Vascular endothelial (VE) cadherin, P-cadherin, E-cadherin and N-cadherin are all expressed in endothelial cells. VE cadherin forms complexes with catenins, some of which link the catenin/cadherin complex to actin microfilaments (5). The cytoskeletal protein vinculin is co-localised with peripheral actin microfilament bundles in the adherens junctions and, via specific actin-binding sites, may facilitate the stable association of actin with the plasma membrane. In addition, adhesion plaques containing vinculin are important sites for anchoring endothelial cells to the sub-endothelium at focal adhesion sites (6). Disruption of these junctions and/or adhesion plaques results in an immediate loss of endothelial integrity due to subtle changes in intercellular adhesion, causing small gaps in the endothelium or frank loss of endothelial cells. Under normal circumstances there is very little turnover of endothelial cells suggesting that endothelial integrity is strictly maintained in the normal resting state by quiescent endothelial cells. Following denuding endothelial injury, the endothelial cell must undergo a transition from a resting cell with a cytoskeleton organised for barrier function to one that promotes cell translocation. Using *in vitro* models it has been established that, in small wounds, adjacent endothelial cells rapidly extrude lamellopodia into the denuded area (7, 8). If repair is incomplete, as in larger wounds, cell migration and eventual proliferation occurs. These responses are accompanied by complex changes in arrangements of the intra-cellular actin microfilament bundles, and stimulated by a

variety of growth factors such as vascular endothelial growth factor and basic fibroblast growth factor (9).

Endothelial control of vascular tone

Endothelial control of vasomotor tone occurs via its production of various vasoactive mediators and it is the net balance of these mediators, i.e. their vasodilatory or vasoconstrictive properties, that determines the local vascular tone and, hence, tissue blood flow. The principal vasodilators produced by the endothelium are nitric oxide (NO), prostacyclin (PGI_2), endothelial-derived hyperpolarising factor (EDHF), adenosine and bradykinin. By contrast, endothelial production of endothelin (ET)-1, angiotensin II (AT II) or endothelial-derived constricting factor (EDCF) results in contraction of vascular smooth muscle cells (VSMC) and, ultimately, vasoconstriction.

Endothelial-derived vasodilators

In 1980, landmark studies by Furchgott and Zawadzki showed that intact endothelium is required for acetylcholine-induced vasorelaxation, and that acetyl-choline stimulates release of a potent vasodilator substance termed endothelial derived relaxing factor (EDRF) (10). It is now known that EDRF is NO and that, in addition to its potent vasodilator properties, NO is an important endogenous inhibitor of leukocyte and platelet adhesion to endothelial cells (11, 12). NO is synthesised constitutively in the endothelial cell from L-arginine by the actions of nitric oxide synthase (NOS) through a five-electron oxidation. Three distinct isoforms of NOS have been identified which catalyse the same redox reaction, but differ in biochemical and structural properties (13). Type 1 NOS is constitutively expressed in

neurons (neNOS) where its activity is regulated by calcium gradients. Type 2 NOS is transcriptionally induced by cytokines, is independent of elevations of calcium and is prototypically expressed in inflammatory macrophages. The type 3 isoform is constitutive in endothelial cells (ecNOS). All NOS isoforms exhibit unique complexity, but common to all is a flavin-containing reductase domain and a haem-containing oxidase domain. This built-in electron transport system is used to oxidise L-arginine to NO and the by-product L-citrulline. Tetrahydrobiopterin, flavin mononucleotide and flavin adenine dinucleotide are cofactors in these reactions while NADPH and molecular oxygen are necessary cosubstrates for optimal NOS activity (14, 15). (Fig. 1.1).

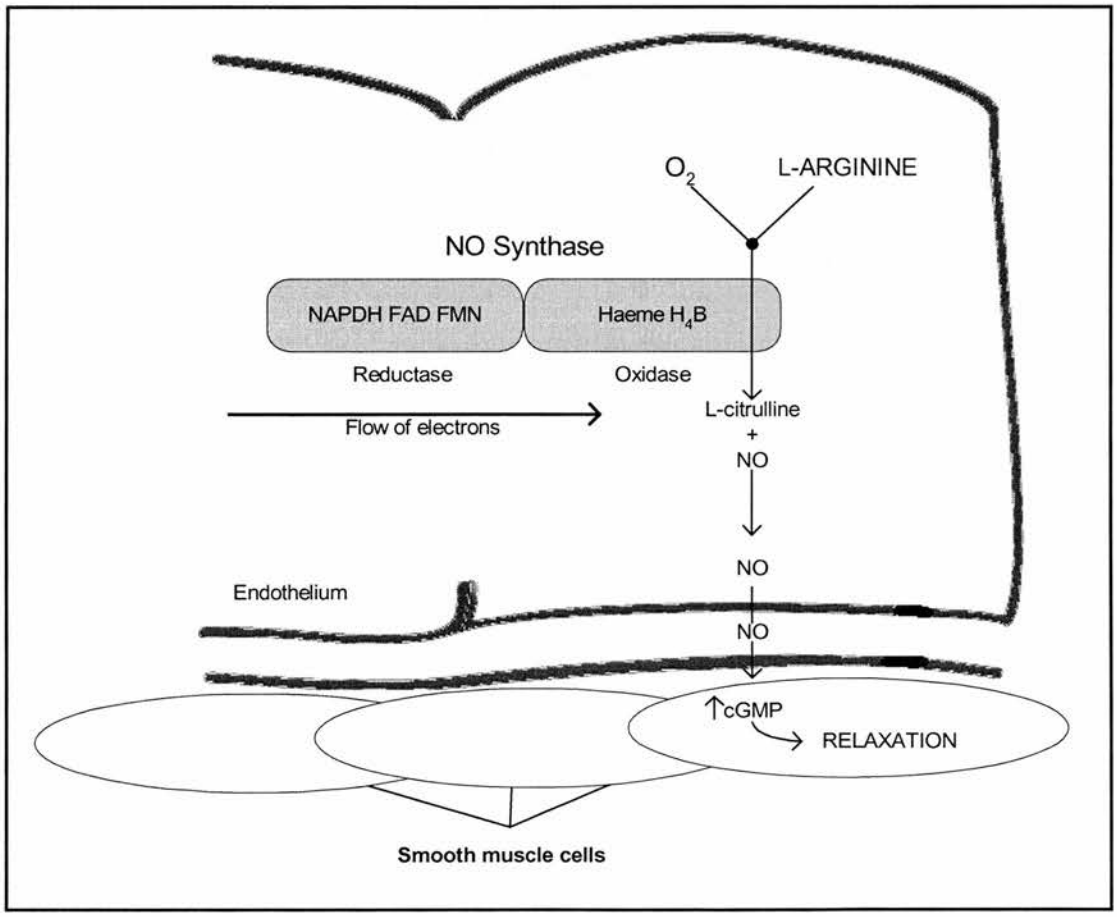


Figure 1.1 NO synthesis (adapted from 16)

ecNOS is dynamically regulated and can increase the production of NO by more than 20-fold within seconds, presumably through the mobilisation of calcium. Chronic increases in blood flow result in a more prolonged release of NO suggesting induction of the NOS gene or of the cofactors necessary for the production of NO by EC. Stimuli that have been shown to increase NOS synthesis include a variety of vasomediators including bradykinin, histamine, catecholamines, substance P, serotonin, thrombin and vasopressin, as well as physical forces such as increased shear stress and cyclic strain (17-19). There does not seem to be any control of NO release as it rapidly diffuses out of the cell, either as free gas or by complexing with carrier proteins, both into the vascular lumen and away from the lumen towards the underlying cells. Before it reaches target cells, however, it may be inactivated by oxygen radicals (O_2^- and OH^-) and iron containing compounds such as haemoglobin (20).

NO stimulates soluble guanylate cyclase in target cells, leading to increases in intracellular cGMP levels (21, 22). In vascular smooth muscle cells (VSMC), cGMP activates cGMP-dependent protein kinase, which phosphorylates myosin light chain kinase and in doing so lowers its affinity for the calcium-calmodulin complex (Fig. 1.2). This results in decreased phosphorylation of myosin light chain, enhanced formation of the comparatively less active, dephosphorylated form of myosin, and VSMC relaxation. Activation of the cGMP-dependent protein kinases can also result in phosphorylation of calcium transporters and complexing of intra-cellular calcium, again resulting in VSMC relaxation.

In addition to NO, endothelial cells synthesise PGI_2 from arachidonic acid via a series of reactions initiated by the enzyme cyclooxygenase (23). PGI_2 acts synergistically with NO to produce vasodilation and relaxes VSMC by the activation of adenylyl cyclase and subsequent increases in intracellular levels of cAMP (Fig. 1.2).

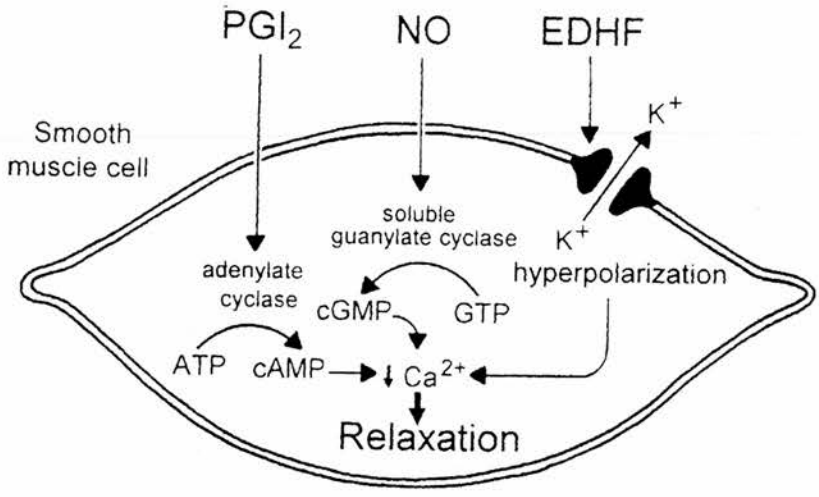


Fig 1.2.
Endothelial-derived vasodilators: action on vascular smooth muscle cells (24).

Several laboratories have described an endothelial-derived vasodilating factor that is distinct from NO or PGI_2. This factor acts by opening calcium-activated potassium channels and hyperpolarizing the smooth muscle membrane and is termed endothelial-derived hyperpolarizing factor or EDHF (3, 25). The identity of EDHF and the possibility of multiple EDHFs has received considerable attention. Recent studies have shown that in animal coronary, cerebral and renal arteries, EDHF is a cytochrome P450 metabolite of arachidonic acid, an epoxyeicosatrienoic acid or EET (26). EETs are released by vasoactive substances such as acetylcholine or bradykinin. Their activation of the calcium-activated K^+ ion channels hyperpolarises

and relaxes smooth muscle (Fig 1.2). It should be pointed out, however, that while some vascular beds possess EDHF activity, they do not produce arachidonic acid metabolites by the cytochrome P450 pathway (e.g. rabbit carotid artery and rabbit aorta), implicating compounds other than EET as stimuli for smooth muscle hyperpolarisation in these vascular beds (27).

The endothelial cells also possesses the requisite machinery to generate adenosine, a potent vasodilator (28). Ecto-ATPase catalyses the hydrolysis of ADP to AMP. AMP is then converted, by the actions of 5'nucleotidase, to adenosine. This pathway not only produces a potent vasodilator but, in addition, the initial hydrolysis step ensures that the platelet-aggregating effects of ADP are regulated. Bradykinin is generated in endothelial cells by the actions of kallikrein on kininogens (29). Bradykinin exerts potent vasodilating actions by stimulating endothelial production of NO, PGI_2 and EDHF. In addition bradykinin promotes release of tPA from endothelial cells, an important mediator of fibrinolysis.

Endothelial-derived vasoconstrictors

The most potent vasoconstrictor produced by endothelial cells is endothelin (ET) (30). ET-1 is the predominant isoform of the endothelin peptide family and regulates vasoconstriction and VSMC growth in tissues through activation of Gi-protein-coupled ET_A and ET_B receptors. ET-1 synthesis in the endothelial cells is enhanced by angiotensin II (AT II) (31), vascular endothelial growth factor (VEGF) (32), cytokines (33), and haemodynamic strain (34). Inhibitors of endothelin synthesis include NO (35), PGI_2 (36), atrial natriuretic peptides (37) and oestrogens (38). The precursor peptide big ET-1 is processed to ET-1 through cleavage by either

endothelin-converting enzymes (ECE), chymase or non-ECE metalloproteinases. ET-1 binding to ET_A and ET_B receptors on smooth muscle mediates potent sustained contraction, in addition to VSMC production of cytokines and growth factors (39). Endothelial expression of angiotensin converting enzyme (ACE) results in the local conversion of AT I to the active peptide AT II (40) (Fig 1.3).

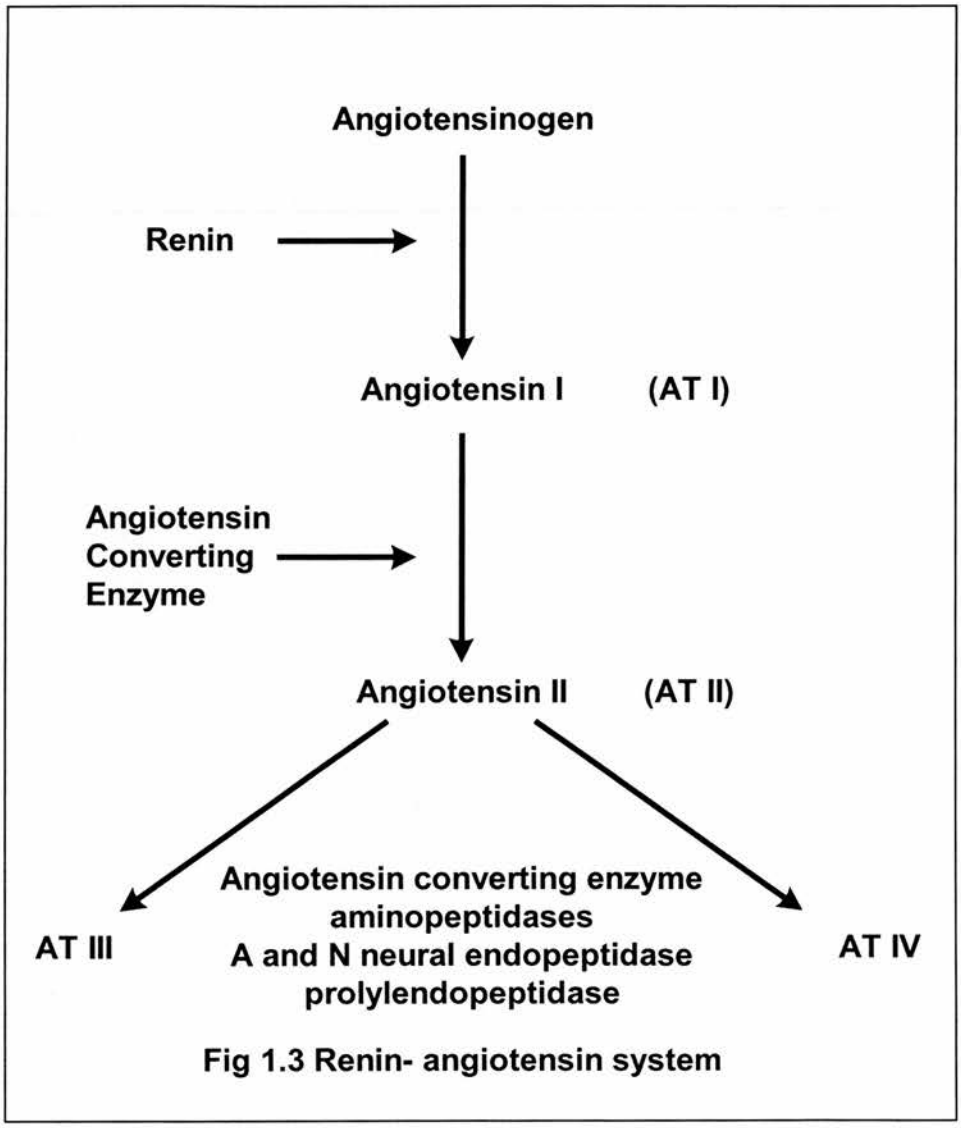


Fig 1.3 Renin- angiotensin system

AT II functions, mainly through the AT II type 1 receptor (AT_1), as an acute vasoconstrictor, regulating systemic blood pressure and vascular tone (41, 42). AT III and AT IV are degradation products of AT II and also possess biological functions. AT III, while possesssing most of the properties of AT II and sharing the

same receptors, is particularly important in brain physiology. AT IV possesses its own receptors distinct from AT_1 and AT_2 and, in contrast to AT II, mediates renal and cerebral vasodilatation although its exact physiological role remains uncertain. Through an independent and parallel pathway, endothelial ACE also inactivates bradykinin, a potent vasodilator.

A number of other factors, termed endothelial derived constricting factors (EDCF), are released by endothelial cells. The composition of these factors is unknown, but likely candidates include cyclooxygenase products of arachidonic acid and other cis-unsaturated fatty acids (43).

Interplay of endothelial-derived vasodilators and vasoconstrictors

The vascular endothelium operates in concert with other systems such as sympathetic neural networks to modulate vascular tone through a balance of vasodilating and vasoconstricting stimuli and inhibitors. Interactions between these vasoactive mediators ensure homeostasis in resting vascular tone as well as rapid responses to the various physiological stimuli e.g. exercise, that necessitate changes in local vascular tone or tissue blood flow (1). Indeed one of the primary manifestations of endothelial dysfunction is the impairment of the endothelium-dependent vasodilator response (44, 45). In addition vasodilators such as NO and PGI_2 have important anti-platelet and growth inhibitory effects (46, 47), while vasoconstrictors such as ET-1 or AT II stimulate growth of the VSMC (48, 49). The anti-platelet and vasorelaxant effects of NO and PGI_2 appear to be synergistic (50), because NO activates cyclooxygenase 1, the constitutive isoform of the enzyme responsible for prostaglandin synthesis from arachidonic acid (51). NO and PGI_2 (39) inhibit

endothelial synthesis of ET-1, while AT II enhances synthesis of this peptide. The vasoconstrictor effects of ET-1 acting through ET_A and ET_B receptors on VSMC are modulated by its binding to *endothelial* ET_B receptors which results in production of the vasodilators NO and PGI_2. ACE promotes vasoconstriction in two principal ways (52). In addition to its involvement in the generation of AT II, ACE also, via an independent and parallel pathway, inactivates bradykinin, thereby preventing bradykinin-induced release of vasodilators (NO, PGI_2 and EDHF). These various interactions, stimulatory and inhibitory, between the endothelial vasoactive mediators, govern the basal vascular tone and mediate rapid physiologic changes in vasomotor tone.

Endothelial anti-thrombotic activity

Haemostasis is the physiologic mechanism that maintains blood in a fluid state within the circulation. Coagulation of blood is mediated by cellular components and soluble plasma proteins and is described in detail in section 1.3.3. (Fig 1.4)

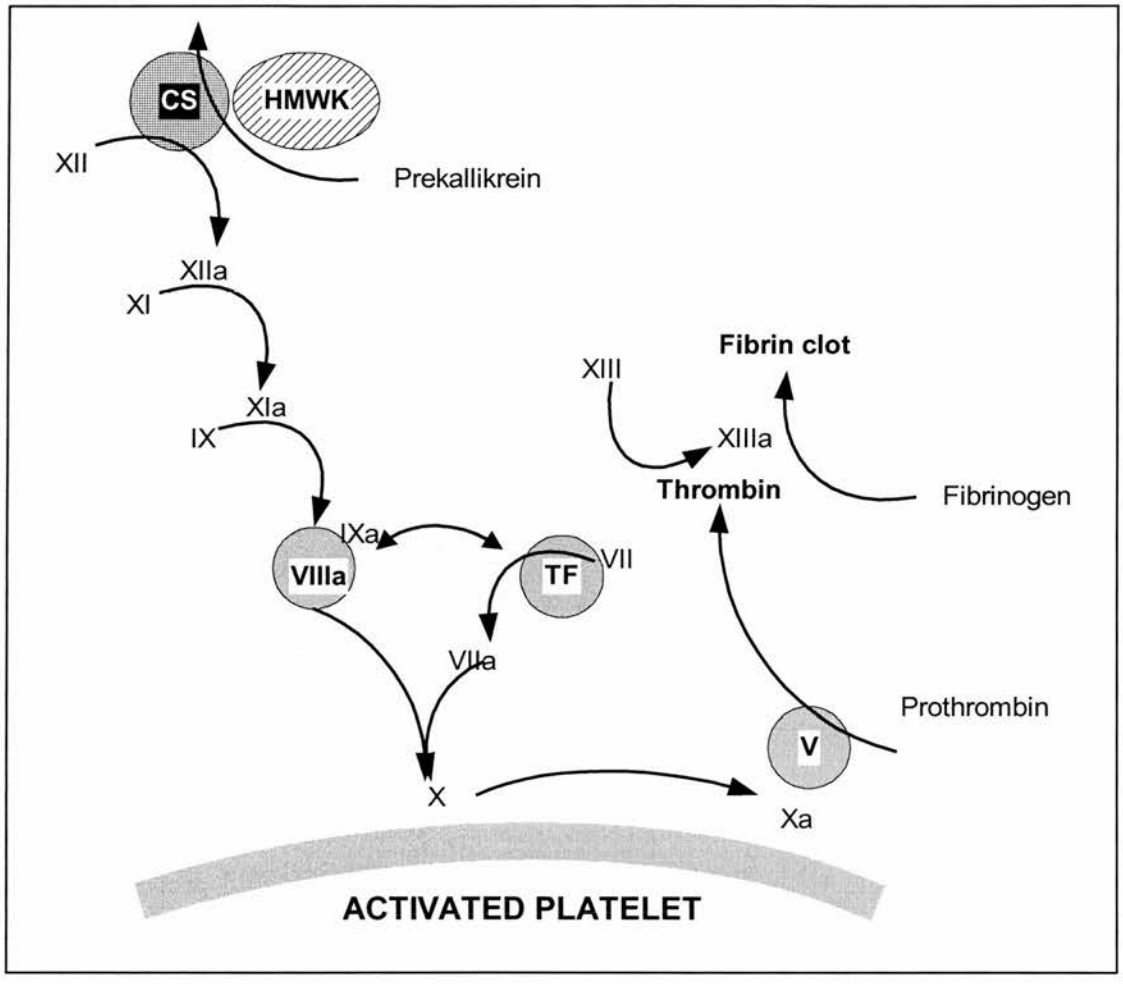


Fig 1.4 Coagulation cascade (53)

The coagulation cascade involves an intrinsic and extrinsic pathway. The intrinsic pathway is initiated by the activation of factor XII by contact with surface (CS), whereas the extrinsic pathway begins with the activation of factor VII by tissue microsomal lipoproteins (TF). Physiologically, the two pathways work together to provide maximum stimulation of factor X which in the presence of factor V activates prothrombin to thrombin. The last step in the cascade is the activation of fibrinogen to fibrin clot with factor XIII acing as a clot stabilising factor.

Endothelial cells maintain haemostasis and inhibit thrombus formation by a number of specific anti-platelet, anti-coagulant and fibrinolytic effects. In addition, the endothelial cell surface bears a negative charge at physiological pH due to heparin-

like sulphated mucopolysaccarides and sialic acid groups of cell surface glycoproteins. Sialic acid groups are also responsible for the negative charge observed on the surface of circulating blood cells. This generates an electrostatic cell repulsion which contributes to the non-reactivity of normal endothelium with circulating blood cells and helps ensure a non-adhesive, non-thrombogenic endothelial surface (54).

1. Endothelial anti-platelet activity

Endothelial production of NO and PGI_2 further contributes to the maintenance of a non-thrombogenic surface. By increasing platelet cGMP levels, NO inhibits both the adhesion of platelets to endothelial cells and their aggregation (55). PGI_2 produces similar effects by increasing platelet cAMP levels (46). As previously discussed, the anti-platelet and vasorelaxant effects of NO and PGI_2 appear to be synergistic (50). Both NO and PGI_2 limit the vasoconstrictor effects of platelet-derived thromboxane A_2 (TxA_2), the major product of platelet cyclooxygenase. Aggregating platelets also release ADP which can be degraded by endothelial ecto-ADPase to AMP and adenosine, another potent inhibitor of platelet function (56).

2. Endothelial anti-coagulant activity

The anti-coagulant activity of endothelial cells is mediated a number of mechanisms. Heparin-sulphate proteoglycans are released by the endothelium and bind tightly to its luminal surface (57). These proteoglycans attach, and subsequently activate, antithrombin III (58). In this manner, the ability of this inhibitor to bind to and neutralise thrombin and the other serine proteases of the clotting cascade is

enhanced. A second anti-coagulant mechanism occurs via the binding of thrombin to thrombomodulin on endothelial cells which alters the substrate specificity of thrombin so that it no longer converts fibrinogen to fibrin and no longer activates factor V (59). Moreover, thrombin binding to thrombomodulin also activates the endogenous anticoagulant protein C (60).

3. Endothelial pro-coagulant activity

Endothelial cells also mediate a number of pro-coagulant effects which, in response to vessel injury, assist in clot formation and excessive blood loss. Endothelial cells release von Willebrand factor (vWF), an adhesive macromolecule which carries factor VIII in plasma (61). vWF facilitates the adhesion of platelets to the exposed sub-endothelium of injured vessels and also mediates inter-platelet interactions in the formation of platelet-fibrin thrombi (62). In addition, endothelial cells express receptors for cell-surface tissue factor, expose critical sites for coagulation-factor complexes and attract platelets and monocytes to sites of tissue /endothelial damage.

4. Endothelial fibrinolytic activity

The dissolution of pre-formed clots, fibrinolysis, is also modulated by the endothelium, through endothelial cell secretion of both plasminogen activators and plasminogen activator inhibitors (63). Tissue-type plasminogen activator (t-PA) is synthesised by vascular endothelial cells and results in the generation of plasmin (from plasminogen), which then acts on fibrin to dissolve clots (Fig 1.5). Endothelial cells express receptors for t-PA that further localise activation of plasminogen to the endothelial cell surface (64). Plasminogen-activator inhibitor (PAI-1) is also

produced and secreted by the endothelial cell, and as its name suggests, inhibits the activation of plasminogen and antagonises fibrinolysis.

Under normal conditions, a delicate balance between the pro- and anti-coagulant properties of the endothelium is achieved by a series of regulatory linking mechanisms. Multiple signals are integrated, so that within any given segment of the

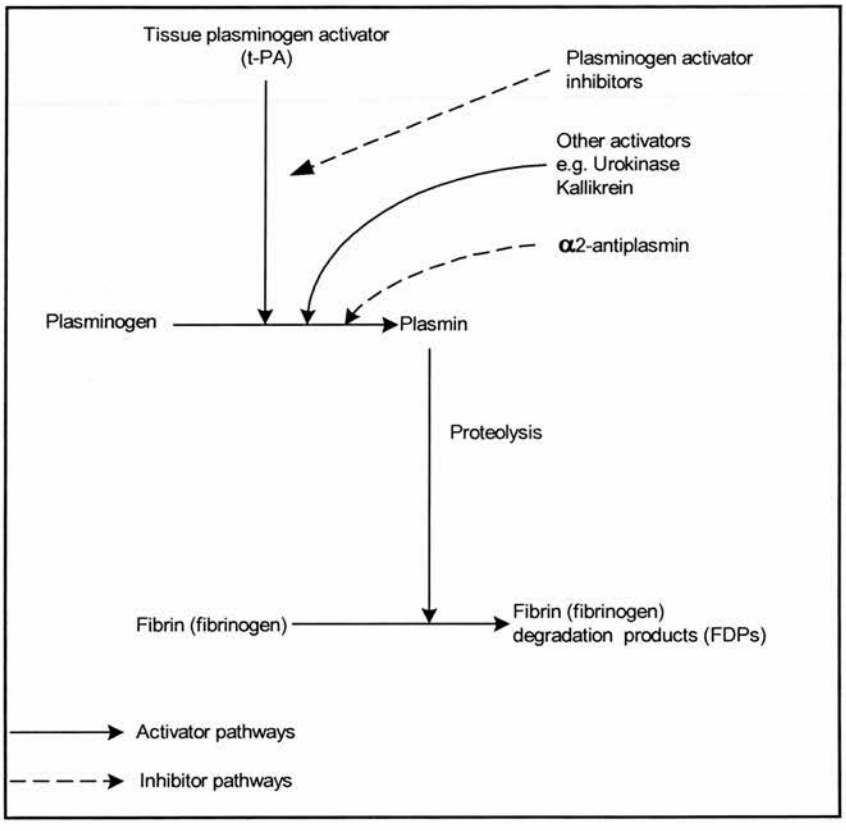


Figure 1.5 Fibrinolysis

vascular tree, the endothelium is capable of shifting the haemostatic balance from moment to moment. This, while providing enormous flexibilty, also makes the endothelium vulnerable to focal dysfunction and pathophysiological disorders (as will be discussed in later sections).

Endothelial growth-regulatory effects

Endothelial cells produce substances that control growth of smooth muscle cells in the vascular intima. In the normal state endothelial release of NO and PGI_2 inhibit VSMC growth (47, 65). These mediators are in themselves growth-inhibitory, and moreover they exert indirect negative regulation in vitro via their inhibition of the release of growth factors such as platelet derived growth factor (PDGF) and epidermal growth factor (EGF) (66). Heparin sulphate proteoglycans on the surface of the endothelial cell also inhibit growth of VSMC *in vitro* (67). Growth promoting products of the endothelial cell include endothelin-1, AT II, basic fibroblast growth factor (bFGF), PDGF, insulin-like growth factor (IGF) and cytokines (68).

The extra-cellular matrix (ECM) is composed of the scaffolding elements of collagens (types I, III, IV and VI) and elastins embedded in a mixture of glycoproteins (e.g. fibronectin) and proteoglycans (e.g. heparin sulphate). These proteins not only provide structural support, but modulate cell function and growth by interacting with growth factors (68). Endothelial cells produce matrix proteins (including collagens, fibronectin, laminin and heparin sulphate), in addition to enzymes which either promote matrix degradation (e.g. gelatinases) or inhibit it (tissue inhibitor of metalloproteinases [TIMP]) (69). Many of these ECM-related proteins are secreted preferentially in the basal direction *in vitro*, and secretion occurs before the cells form a coherent layer. During periods of cell proliferation, such as angiogenesis or vascular remodelling, initial proteolytic degradation of the ECM is nececessary, followed by synthesis of ECM components and reconstruction of the matrix scaffolding. It appears that endothelial cells are central to the

homeostatic control of these processes by the elaboration of the various proteases and their inhibitors (69).

1.1.2. Vascular smooth muscle cells

Blood vessels are comprised of three layers: the intima, media and adventitia. The intima, the innermost layer, is made up of one cell type – the endothelium – which lines the luminal surface. Intimal smooth muscle cells can also be found occasionally, but their frequency depends on species, age and pathology. The media is composed of multiple layers of vascular smooth muscle cells (VSMC) and is separated from the intima by an internal elastic lamina. The outermost adventitial layer consists of loose connective tissue and contains blood vessels and nerves. The principal function of the VSMC is contraction and these cells have developed a repertoire of appropriate contractile proteins, agonist receptors, ion channels and signal-transducing molecules to carry out this specialised function. VSMC however are also capable of a number of other functions that vary at different developmental stages, during vascular repair and in vascular disease (70). Fully differentiated VSMC in mature blood vessels proliferate at extremely low rates and produce only small amounts of ECM matrix proteins. These processes are greatly accelerated during development of the vascular system, during vessel remodelling, following vessel injury and in atherogenesis (as will be discussed in section 1.3.1) (71).

A given VSMC can rapidly, and reversibly, acquire a broad spectrum of different phenotypes (in terms of contractility, secretion of ECM components, proliferation and migration) in response to these different physiological or pathological stimuli. If an artery is injured, for example, some VSMC must be recruited to repair that injury, while at the same time, the contractile function of the blood vessel must be maintained for normal cardiovascular homeostasis (72). Endothelial-VSMC interactions with the subsequent release of endothelial mediators (growth-inhibiting

and growth stimulating), neuronal influences (such as sympathetic innervation) and haemodynamic forces may all influence the phenotypic expression of VSMC. The cellular and molecular mechanisms controlling the coordinate expression of genes determining these various phenotypes are still poorly understood (70).

Mature VSMC express unique isoforms of a variety of contractile and cytoskeletal proteins (actin, myosin, calponin, caldesmon, vinculin, tropomyosin, desmin and vimentin) that are important for their differentiated function. The most abundant of these is smooth muscle α-actin.which is the earliest known marker of differentiated VSMCs expressed during vasculogenesis (73). There is also evidence that VSMC from different vascular sites (and indeed within the same vessel) display phenotypic and functional heterogeneity, with cells differing in size, organelle content, the expression of cytoskeletal and contractile proteins and protein isoforms (74). This has led to the concept of *cell diversity* which suggests that the vascular media is a mosaic of functionally and morphologically unique cell types. If true, this would explain regional variations in the response of vascular beds to vasoactive mediators and how vessels localise remodelling in response to injury or hypertension.

1.1.3. Pericytes

Pericytes (also known as Rouget or mural cells) are associated abluminally with all vascular capillaries and post-capillary venules. Most studies on pericyte function have focused on retinal microvascular pericytes and mesangial cells. Differences in pericyte morphology and distribution among vascular beds suggest vessel or tissue-specific roles including phagocytosis, regulation of new capillary growth and control of capillary blood flow. Evidence has also been provided which indicates that pericytes behave both as multipotent mesenchymal cells and specific precursors to VSMC (75).

Each pericyte possesses a cell body with a prominent nucleus and a small amount of surrounding cytoplasm (76). Protruding from the cell body are long processes which parallel the long axis of the capillary and taper to smaller processes which encircle the capillary wall. Processes penetrate the surrounding ECM to directly contact the underlying endothelium, as well as interdigitating among neighbouring cells. Pericytes are embedded within a basement membrane that surrounds the capillary tubes. *In vitro* evidence suggests that both endothelial cells and pericytes contribute to basement membrane formation (77). Based on their location and their complement of muscle cytoskeletal proteins (including α-smooth muscle actin, fibronectin, myosin, tropomyosin), pericytes have been proposed to play a role in the regulation of blood flow. *In vitro* studies demonstrating the contractile ability of pericytes support this concept (78, 79). The marked similarity of pericytes to VSMC has led to the theory that these cells are specific precursors of VSMC (80). A study of mesenteric growth in rats, using intravital video and electron microscope analyses, provided evidence that fibroblasts transform into capillary pericytes which, in turn,

become VSMC (81). These authors speculate that as capillaries are remodelled into larger vessels to meet an increased functional demand, pericytes are differentiated to become true VSMC as needed. These cells appear to be multipotent, capable of becoming adipocytes, osteoblasts and phagocytes (75). They are also thought to be structurally similar to mesangial cells in the glomerulus, as discussed below.

Pericytes have been speculated to play a role in the regulation of capillary growth. The exact mechanisms by which these cells promote vessel formation have yet to be elucidated, but observations indicate that the endothelium can recruit undifferentiated mesenchymal cells and direct their differentiaton into pericytes in microvessels and VSMC in large vessels (82). Communication between endothelial cells and pericytes (or their precursors) may take many forms. Bi-directional extracellular exchange of soluble mediators takes place between the endothelial and mural cells. Amongst the endothelially-derived mediators, candidate effectors for pericyte recruitment are PDGF, bFGF and heparin-binding epidermal growth factor (HB-EGF) which is similar to epidermal growth factor (83). Once pericyte precursors have been recruited to the endothelial cell, the two cells make contact via cell-adhesion molecules (CAMs), substrate adherence molecules, the ECM itself and gap junctions (84). Although few data exist, it has been speculated that pericyte contacts, in addition to the release of growth factors, may contribute to the growth and differentiation of pericytes and smooth muscle cells. It appears that cell-cell contact, for example, is necessary for the functioning of the cytokine TGF-β (85). This cytokine is secreted *in vitro* in a latent form by both cell types and activated on endothelial-pericyte contact. TGF-β induces the expression of α-SM actin (a VSMC marker) both in myofibroblasts and in pericytes (86). Further, evidence that this cytokine is involved

in the growth and differentiation of both endothelial cells and pericytes during vessel formation was provided by the observation that mice lacking TGF-β display a suppression of endothelial cell differentiation and insufficient vascular development (87). The role of endothelial cell-pericyte contacts is almost certainly not limited to vessel formation, and cell-cell interactions may also be vital to the maintenance and functioning of the quiescent vessel. Endothelial/pericyte synthesis and secretion of mediators which both stimulate and inhibit pericyte growth and differentiation almost certainly occurs *in vivo*. The *in vitro* observation that conditioned media from confluent endothelial cells inhibits the growth of pericytes supports this concept (88).

Mesangial cells

Mesangial cells (MC), together with their extracellular matrix material, constitute the mesangium and are separated from the glomerular capillary lumen by the endothelium. As noted earlier, it is generally accepted that glomerular MC are, in effect, specialised pericytes (89). These cells possess many of the functional properties of smooth muscle cells e.g. they provide structural support for the glomerular capillary loops, produce growth factors and extra-cellular matrix components and via their contractile properties, modulate glomerular haemodynamics (90).

Mesangial cell structure

MCs are irregular in shape with numerous cytoplasmic processes arising from the cell body (91). These processes run toward the perimesangial glomerular basement membrane (GBM) and can extend around the capillary lumen, frequently insinuating themselves between the basement membrane and the overlying endothelium. An

extensive array of microfilament bundles, composed in part of actin, α-actinin and myosin, are situated predominantly in the mesangial cell processes. These run mostly transversely or diagonally from one side of a process to another and terminate by connecting with the GBM either directly, or indirectly through the interposition of extracellular microfibrils. The GBM plays the role of a "skeleton" in this system, with the extracellular myofibrils functioning as "microtendons." The MC is also surrounded by a matrix material that differs from the glomerular GBM in its "loose" texture and the presence of small bundles of fine fibers. This matrix contains sulphated glycosaminoglycans as well as fibronectin and laminin and, in addition to providing structural support for the glomerular capillary tuft, has been implicated in the adhesion, migration, proliferation and differentiation of MCs.

Mesangial cell: contractile functions

The properties and functions of the MC have been defined largely from cell culture studies. Contraction *in vitro* occurs in response to a variety of vasoactive agents, including angiotensin II, vasopressin, norepinephrine, TxA_2, leukotrienes and platelet activating factor (PAF) (92). Furthermore, receptors for these mediators have been demonstrated on the MC. Similar to smooth muscle, the signal transduction pathways and ion-selective channels regulating MC contraction are dependent on the voltage-gated Ca^{2+} influx. Relaxation *in vitro* occurs in response to such agents as prostaglandin (PG) E_2, atrial natriuretic peptides and dopamine, and involves increases in cGMP in addition to the activation of plasmalemmal K^+ channels which hyperpolarise the membrane potential and inhibit voltage-gated Ca^{2+} entry (93). It is generally held that MCs in situ will also respond to vasoactive substances although this is based on indirect evidence derived from *in vitro* studies. The contractile

properties of the MC suggest a regulatory role in role glomerular haemodynamics. It has been speculated that contractile forces in the mesangium counterbalance the ultrafiltration pressure, preserving the architecture of the capillary network (91). An alternative theory proposes that MC contraction/relaxation can regulate the glomerular filtration rate (GFR) by modulating the surface area of the capillaries (94).

Mesangial cell: immune functions

In addition to their role in the maintenance of the structure and function of the glomerulus MC are capable of macromolecular uptake and the generation of autocoids and cytokines (89). Although evidence suggests that much of the phagocytic ability of the mesangium resides in bone-marrow derived resident monocyte/macrophages, it appears that MCs are themselves capable of phagocytosis. *In vitro*, MC phagocytose opsonised zymosan in association with the production of prostaglandins, reactive oxygen species and lipoxygenase products (95). Phagocytosis of immune complexes by cultured MC also occurs and is accompanied by the production of PGE_2 and PAF (96). MCs express Fc receptors for IgG and receptor expression is upregulated *in vitro* by interferon(IFN)-γ, cAMP and monocyte/macrophage colony stimulating factor (M-CSF-1) (97). In addition, uptake of immune complexes is acutely increased by AT II and inhibited by cAMP. These latter changes are independent of Fc receptor number, but are accompanied by changes in the MC cytoskeleton. Uptake of tracers such as ferritin, colloidal carbon, globin, aggregated proteins and immune complexes into the mesangium have been demonstrated in animal models *in vivo*, but there is some controversy as to whether

phagocytosis in these studies has been mediated by MC or resident bone-marrow derived macrophages (92).

Many forms of both immune and non-immune glomerular injury are accompanied by the glomerular recruitment and activation of macrophages. This requires prior expression of cellular adhesion molecules, in addition to the generation of chemokines, on resident cells. Stimulation of mesangial cells *in vitro* with immune complexes and pro-inflammatory cytokines such as tumour necrosis factor(TNF)-α or interleukin(IL)-1 results in the appearance of adhesion molecules on MC (intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1)), in addition to the elaboration of chemokines such as monocyte chemotactic protein(MCP)-1, IL-8, *r*egulated upon *a*ctivation, *n*ormal *T* cell expressed and *s*ecreted (RANTES) and macrophage inflammatory protein-(MIP)-2 (98, 99). Indeed MCP-1 expression is increased in MC in response to a variety of stimuli including IFN-γ, thrombin, low density lipoprotein (LDL) and phorbol esters (100). Upregulation of chemokine expression in MC involves, at least in part, the activation of the transcription factor NF-κB through protein tyrosine kinase-dependent mechanisms. The ability of the mesangial cell to release not only chemokines, but a wide range of growth factors and cytokines (such as PDGF, insulin-like growth factor(IGF)-1, IL-1 and IL-6) suggests that, rather than a passive target for the action of infiltrating cells, MC are themselves important participants in glomerular injury, as will be discussed in later sections (2.5, 2.6) (101).

Mesangial cells: proliferation and extracellular matrix production

MC elaborate a number of autocrine growth factors which both regulate cell function and the production of extracellular matrix. MC can also respond to growth factors released from neighbouring glomerular cells or from infiltrating cells and platelets (102). Such responses include MC hypertrophy and proliferation and matrix metabolism. PDGF mediates predominantly cell proliferation, while TGF-β mediates MC matrix expansion. ECM has been implicated in the adhesion, migration, proliferation and differentiation of MCs and indeed its composition may play an important role in the regulation of MC apoptosis (103).

Macrophage conditioned medium promotes a series of responses in cultured mesangial cell. These include the upregulation of the MC genes and/or proteins for the ECM components fibronectin, laminin and collagen IV, the modulators of matrix turnover TIMP-1 and transin, in addition to genes for the growth factors TGF-β and PDGF (104). With regard to fibronectin, treatment of MC with TNF-α, IL-1β or TGF-β alone induces only modest increases in fibronectin protein levels. Combining TGF-β with TNF-α or IL-1β however results in a marked synergistic increase in fibronectin mRNA and protein levels, accompanied by down-regulation in expression of the matrix proteinase transin and an increase in TIMP-1 expression. These data suggest that complex interactions take place between cytokines in the glomerulus to modulate MC matrix synthetic and degradation pathways. In theory, alterations in the local cytokine network could potentially alter MC matrix homeostasis and result in both MC proliferation and excess glomerular deposition of extracellular matrix.

In summary, the MC has several functions in the normal glomerulus, - producing growth factors to allow a normal cell turnover, providing structural support via the secretion of ECM, maintaining glomerular haemodynamics (by both contraction and release of vasoactive mediators) and aiding in the clearance of immune complexes. These functions are presumably tightly regulated and beneficial to the host. Evidence now exists that MCs, activated during glomerular injury/inflammation, proliferate and undergo a phenotypic modulation in which they markedly up-regulate their expression of smooth muscle like proteins e.g. α-smooth muscle actin and produce excess amounts of extra-cellular matrix. If this response is limited then it is potentially reversible, otherwise glomerulosclerosis results. These consequences of mesangial cell activation will be discussed in more detail in later sections.

1.2. Mechanisms and consequences of vascular cell activation in "appropriate" host defence

Mobilisation of the inflammatory response is primarily intended to neutralise the initiating and "injurious" stimulus, limit tissue damage and initiate healing and repair. Inflammation is a complex, highly orchestrated process involving many cell types and molecules, some of which initiate, amplify or sustain the process, some of which attenuate it, and some of which promote its resolution. The importance of the vascular endothelium in regulating these processes is well-established. Normally the endothelium is in an "unactivated" state. At sites of injury or inflammation, however, local endothelial "activation" occurs, either in response to the injuring stimulus itself, in response to the inflammatory cytokines generated as a consequence of this injury or in response to leukocyte-endothelial cell adhesion (105). Activated vascular endothelium is hyperadhesive for leukocytes due to the up-regulated expression of a number of membrane-associated leukocyte-endothelial cell adhesion molecules (CAM) (106). It also secretes chemotactic factors (107) which attract peripheral blood leukocytes and soluble factors which promote coagulation (108). Endothelial activation occurs over a period of minutes to hours and is dependent on mobilisation of stored intracellular proteins, such as P-selectin and vWF (109), and up-regulation of gene expression and *de-novo* protein synthesis.

Inflammatory cytokines are intercellular signalling peptides produced by activated cells in response to injurious stimuli such as endotoxin, immune complexes, and physical and chemical injury (110). Most cytokines have multiple sources, multiple targets and multiple functions. The cytokines that are produced during, and

participate in, inflammatory processes are the chief stimulators of the acute phase response (notably interleukin (IL)-6, IL-1β, tumour necrosis factor (TNF)-α and interferon (IFN)-γ). This response accompanies inflammation and is characterised by a large number of changes, distant from the inflammatory focus, involving many organ systems. The acute phase response is not discussed in detail here, but has been reviewed extensively elsewhere (111). Cytokine production also promotes the activation of vascular endothelium, leukocytes, platelets, fibroblasts and smooth muscle cells, thereby initiating and propagating the cascade of vascular, cellular and humoral events which comprise the inflammatory response.

1.2.1. Leukocyte trafficking

The histological hallmark of acute inflammation is the infiltration of interstitial tissues by peripheral blood leukocytes – initially by neutrophils and, later, mononuclear leukocytes. The movement of immune cells from the blood vessel lumen into tissue occurs via a coordinated process involving directed movement up concentration gradients of chemoattractants, adhesion to the endothelium, penetration of endothelial cell tight junctions and finally migration through the endothelium, basement membrane and extravascular tissue (112). The infiltrating leukocyte population is, therefore, defined by the nature of the chemotactic cytokines (chemokines) secreted by the cytokine-activated tissue cells surrounding the assault and by the infiltrating cells themselves, and by the profile of adhesion molecules expressed by leukocytes and resident tissue cells. The current model of leukocyte trafficking holds that, following their chemotactic movement to the endothelium surrounding the site of injury/inflammation, initial 'loose' tethering (so-called "rolling") of circulating leukocytes is brought about by interaction between the

selectins and various carbohydrate-containing counter-receptors located on immune cells. This selectin-mediated adhesion in turn facilitates the immobilisation of leukocytes - by the interaction of endothelial immunoglobulin-like adhesion molecules (notably intercellular adhesion molecule-1, ICAM-1 and vascular cell adhesion molecule VCAM-1) with leukocyte integrins - and the eventual movement of these cells from the vessel lumen to the extravascular space (diapedesis) (Fig 1.6) (113). The latter process is facilitated in some systems by adhesion of leukocytes to endothelium and basement membrane through homotypic and heterotypic adhesion processes involving another immunoglobulin-like adhesion molecule PECAM-1.

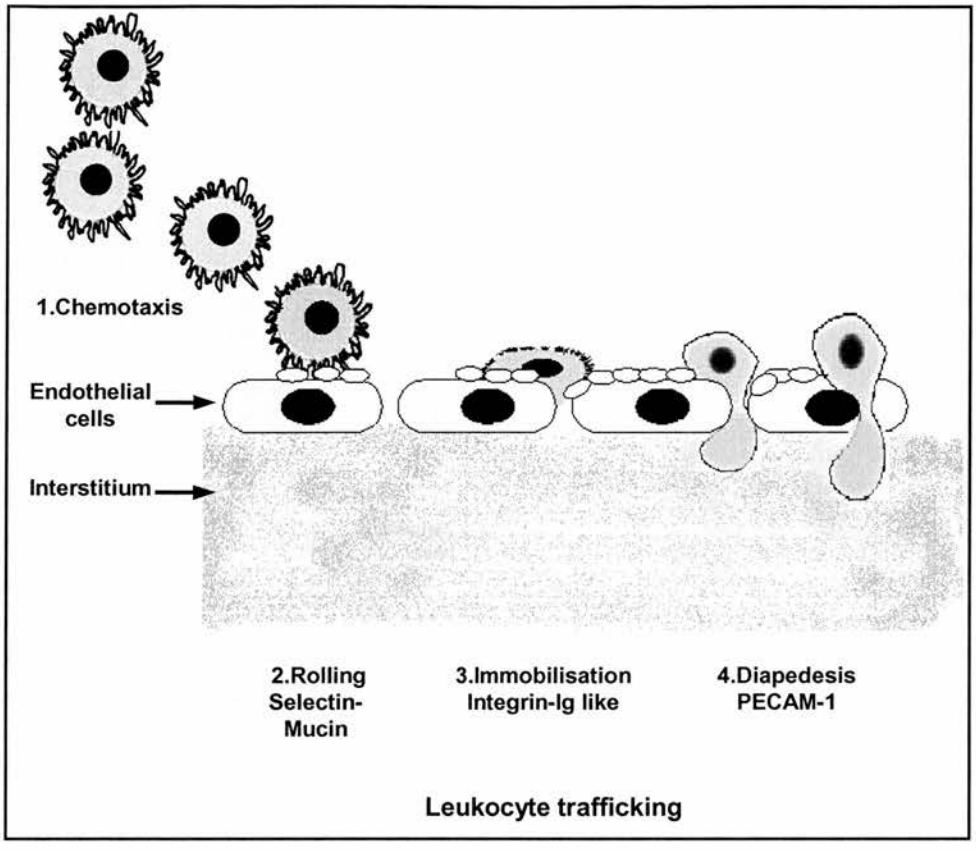


Fig 1.6

Following chemotaxis (1), initial and attachment and rolling of leukocytes is mediated by selectins binding their counter receptors (2). Neutrophils roll and decelerate on both activated endothelium as well as other adherent neutrophils. Stable adhesion is mediated by adhesion between integrins present on the leukocyte surface and Ig on the surface of activated endothelium (3). Transendothelial migration is mediated by PECAM-1-PECAM-1 interactions as well as integrin-Ig bonds (4). Chemokines may also promote diapedesis by activating leukocyte integrins or inducing chemotaxis. Chemokines also direct the interstitial migration of transmigrated leukocytes and induce leukocyte activation. Extravasated leukocytes may in turn generate more chemoattractants, thereby amplifying the recruitment process.

1.2.2. Leukocyte-endothelial adhesion molecules

Data derived from both *in vitro* (isolated leukocytes binding to monolayers of cultured endothelial cells) or purified and immobilised adhesion proteins (114) and *in vivo* (intravital microscopic examination of venules and gene "knock-outs") (115) models of leukocyte-endothelial cell adhesion have revealed the relative contributions of different leukocyte and endothelial cell adhesion molecules (CAMs) to the adhesion responses elicited by various inflammatory stimuli. The various leukocyte-endothelial adhesion molecules and their corresponding ligands are shown in the table below (Table 1.1).

Family	Members	Cluster designation	Cellular distribution	Counter-receptor / ligand
Selectins	E-selectin	CD62E	Endothelium	PSGL-1, ?other carbohydrate-bearing structure(s)on leukocytes
	L-selectin	CD62L	Leukocytes	PSGL-l, ?inducible carbohydrate-bearing structure(s)on endothelium
	P-selectin	CD62P	Endothelium, platelets	PSGL-1, ?other carbohydrate-bearing structure(s) on leukocytes
Mucin-like	PSGL-1	CD162	All blood leukocytes	E-, L- and P-selectin
β_1 integrins	$\alpha_4\beta_1$	CD29/CD49d	Monocytes, lymphocytes	VCAM-1, fibronectin
β_2 integrins	LFA-1	CD11a/CD18	Leukocytes	ICAM-1, ICAM-2, ICAM-3
	Mac-1	CD11b/CD18	Monocytes, neutrophils	ICAM-1, fibrinogen, C3b
	P150,95	CD11c/CD18	Monocytes, neutrophils	C3b,fibrinogen
		CD11d/CD18	?macrophages ?dendritic cells	?ligand
$\beta_7$integrins	$\alpha_4\beta_7$	CD49d/CD-(LPAM-1)	Lymphocytes	VCAM-1, fibronectin, MAdCAM-1
Ig	ICAM-1	CD54	Endothelium, leukocytes, epithelial cells, fibroblasts, other cell lines	LFA-1, Mac-1
	ICAM-2	CD1O2	Endothelium	LFA-1
	ICAM-3	CD5O	Leukocytes	LFA-1
	VCAM-1	CD1O6	Endothelium, smooth muscle cells	$\alpha_4\beta_1$, $\alpha_4\beta_7$
	PECAM-1	CD31	Endothelium, leukocytes, platelets	PECAM-1

Table 1.1 Cell adhesion molecules (105)

Selectins

Selectins are trans-membrane glycoproteins which support leukocyte-endothelial and leukocte-platelet rolling/adhesion. Three selectins have been identified and named according to their cell of original discovery: P-selectin (platelet selectin), E-selectin (endothelial cell selectin) and L-selectin (leukocyte selectin) (116, 117). L-selectin is constitutively expressed by most leukocytes but not by other cell types. P-selectin is expressed on the surface of activated endothelial cells and platelets (109). The majority of P-selectin is stored in the Weibel-Palade bodies of endothelial cells and in α-granules of platelets. Mobilisation of P-selectin to the cell surface occurs on activation where it supports adhesion of granulocytes, monocytes and some lymphocyte subsets. E-selectin is expressed, probably exclusively, by cytokine-activated endothelial cells and, like P-selectin, supports the rolling of granulocytes, monocytes and some T lymphocytes (117).

The physiological counter-receptors of selectins have not been fully elucidated, but appear to be carbohydrate structures presented by glycoproteins on the surface of vascular endothelium, leukocytes and platelets (118). The best characterised selectin counter receptor is P-selectin glycoprotein ligand (PSGL)-1 which is present on neutrophils, monocytes and lymphocytes and binds P-selectin and possibly also L- and E-selectin (119). Neutrophil-neutrophil adhesive reactions that occur during neutrophil aggregation are also mediated by PSGL-1-L-selectin bonds. L-selectin also binds to the mucin GlyCAM-1 expressed on high endothelial venules of lymph nodes and this interaction may regulate normal recirculation of leukocytes through lymph nodes *in vivo* (120).

Integrins

Selectin-mediated interactions result in loose tethering and rolling of leukocytes on endothelium and facilitate their subsequent immobilisation by the interaction of leukocyte integrins with immunoglobulin-like molecules on the endothelium. Integrins are transmembrane glycoproteins composed of non-covalently linked α and β subunits and mediate diverse cell-cell and cell-matrix interactions. They are classified according to their β chains and eight β chains have been identified to date. The integrins associated with leukocyte adhesion belong to the β1, β2 and β7 subfamilies (121).

Members of the β2 subfamily contain one of four different α chains designated CD11a, CD11b, CD11c and CD11d that are coupled to a common β chain, CD 18. The heterodimer CD11a/CD18 is expressed on the surface of most leukocytes and interacts with ICAM-1 and ICAM-2 on endothelial cells to cause firm adhesion (112). CD11b and CD11c, expressed by granulocytes and monocytes, are stored in granules and on phagocyte activation are rapidly mobilised to the cell surface by fusion with the cell membrane (122). Activation by inflammatory mediators such PAF or cytokines, such as TNF-α, result in a marked increase (10-30 fold) in expression of these integrins on the cell surface. CD11b/CD18 interacts with endothelial ICAM-1, while the ligand for CD11c/CD18 remains uncertain. A role for the recently described CD11d/CD18 in leukocyte recruitment has not yet been established (123). The adhesion of unstimulated leukocytes to endothelial cells is mediated by CD11a/CD18-ICAM-1 interactions, while activated leukocytes bind to endothelial ICAM-1 by both CD11a/CD18 and CD11b/CD18. The biological

importance of the β2 integrins in the immune response was demonstrated by identification of their deficiency in humans. Heterogeneous mutations of the CD18 gene result in defective surface expression of the entire CD11/CD18 complex. This leukocyte adhesion deficiency (LAD) is characterised by defective leukocyte trafficking, recurrent severe infections and impaired wound healing (124).

Heterodimers of the β1 and β7 subfamilies also contribute to recruitment of different leukocyte populations. The β1 integrin α4β1, also known as very late antigen-4 (VLA-4) mediates the adhesion of lymphocytes, monocytes, eosinophils and natural killer cells to activated endothelial cells expressing the counter-receptor VCAM-1 (125). The β7 integrin α4β7 is found on lymphocytes that colonise the gut and its associated lymphoid tissue This mediates the normal homing of lymphocytes to Peyer's patches, but, via its interaction with endothelial VCAM-1, it also facilitates leukocyte adhesion under conditions of inflammation (126).

Immunoglobulin superfamily

Five members of the immunoglobulin superfamily act as adhesion molecules: ICAM-1, ICAM-2, VCAM-1, platelet-endothelial cell adhesion molecule-1 (PECAM-1) and mucosal address in cell adhesion molecule-1 (MAdCAM-1) (127). ICAM-1 is a glycoprotein constitutively expressed by many cell types including vascular endothelium, fibroblasts, mesangial cells, epithelial cells and skeletal myocytes. Its expression on these cells is up-regulated several fold by endotoxin and inflammatory cytokines. This up-regulation varies from one vascular bed to another and, in general, those beds with high constitutive expression like the lung show less up-regulation than those with low constitutive expression (e.g. skeletal muscle or

heart) (113). Cytokines can also induce ICAM-1 expression on other cell types such as VSMC, mesangial cells and epithelial cells (112). ICAM-1 is also expressed by lymphocytes and indeed lymphocyte-lymphocyte adhesion via ICAM-1-CD11a/CD18 binding may facilitate other lymphocyte functions such as antigen recognition, lymphocyte co-stimulation and cytotoxicity. A soluble isoform of ICAM-1 (sICAM-1) can be detected in the plasma during inflammation and represents shed fragments of endothelial ICAM-1 (128). In a variety of inflammatory and immune conditions serum levels of sICAM-1 have been found to correlate with disease activity. ICAM-2 is constitutively expressed by endothelial cells and lymphocytes and its expression by the endothelial cell is not influenced by the degree of endothelial activation. ICAM-2 binds to CD11a/CD18, but with a lower affinity than ICAM-1 (129).

VCAM-1 mediates the trafficking of monocytes and lymphocytes via binding to the β1 and β7 integrins (113). It is constitutively expressed at low levels predominantly in endothelial cells, but also in VSMC and dendritic cells. Upregulation of VCAM-1 expression in these cells, and others such as mesangial and tubular epithelial cells, occurs in response to cytokine stimulation.

PECAM-1 is constitutively expressed by vascular endothelium (mainly at tight junctions), neutrophils, monocytes, some lymphocytes and platelets (130). It mediates endothelial cell-cell adhesion, myeloid (neutrophil and monocyte) cell-platelet adhesion and myeloid cell-endothelial adhesion by PECAM-1-PECAM-1 interactions. PECAM-1 was originally implicated in the transmigration of leukocytes between intercellular junctions of vascular endothelium (131). However, some

memory T-cells do not express PECAM-1 but are able to transmigrate successfully and it now appears that this process is more complex than previously appreciated. *In vitro* neutrophil adhesion to activated endothelial monolayers alters the molecular composition and organisation of the endothelial cell VE-cadherin complex (probably through selective proteolysis) which has been implicated in cell-cell adhesion and cell-to-cytoskeletal integrity (132). This may facilitate the subsequent transmigration of neutrophils. Another study challenged the long-dominant paradigm that transmigrating neutrophils pass between endothelial cells and provided evidence that in dermal venules neutrophils, in response to the chemoattractant fMLP, emigrated by a transcellular route through both endothelial cells and pericytes (133). It remains to be seen whether this observation can be extended to other inflammatory cells, stimuli or vascular beds.

Platelet adhesion molecules

Platelets are crucial to the body's normal inflammatory response because of their ability to form haemostatic platelet plugs very rapidly at the site of vascular injury, in addition to their release of growth and chemotactic factors. The surfaces of platelets are densely decorated with β1 and β3 integrins which mediate both adherance to the subendothelial matrix exposed by vascular injury and aggregation in response to activation by various agonists such as ADP, adrenaline or thrombin (105). Platelet adhesion is mediated by α2β1 (binds collagen), α5β1 (binds fibronectin), α6β1 (binds laminin) and αIIbβ3 (also called gpIIb/IIIa). Platelet aggregation, on the other hand, is mediated exclusively by the glycoprotein gpIIb/IIIa (134).

1.2.3. Chemokines

Classical chemoattractants such as leukotriene B_4, platelet activating factor and complement components (e.g.C5a and its cleavage product c5a $_{des\ arg}$) are generated during the acute inflammatory response and mediate the migration of leukocytes to sites of tissue injury (105). Chemokines (*chemo*tactic cyto*kines*) are a family of peptides with potent leukocyte attractant and activating properties that exhibit restricted and selective patterns of leukocyte attraction. Their production at the inflammatory site therefore, both by resident cytokine-activated tissue cells and infiltrating cells, defines the nature of the infiltrating leukocyte population (135).

Forty or so chemokines have been described to date and have been divided into four subfamilies according to the relative position of the first two of their four cysteine residues (135). In the C-C family cysteines are adjacent, while in the C-X-C family they are separated by one amino acid. The only known C-chemokine family member is lymphotactin, with two instead of four cysteines, while fractaline, with three amino acids between the first two cysteines at the N-terminal of a mucin structure, is the only known CX_3C chemokine. Chief among the C-C family are the monocyte chemoattractant proteins (MCP), *r*egulated upon *a*ctivation, *n*ormal *T* cell expressed and *s*ecreted (RANTES) and macrophage inflammatory protein (MIP) attracting monocytes, T cells, natural killer cells and some granulocytes but not neutrophils. Principal C-X-C chemokines include interleukin(IL)-8 and growth related oncogene (GRO) which, until recently, were thought to attract neutrophils but not monocytes. There is increasing evidence, however, that, in certain instances these chemokines may play a role in monocyte/macrophage recruitment e.g. to the atherosclerotic plaque, and this is discussed in detail in section 1.3.1 (136). In contrast to the

majority of C-X-C chemokines which attract predominantly neutrophils, three C-X-C proteins show a different spectrum of activity. Interferon-inducible protein of 10kd (IP-10) and monokine induced by interferon-γ (Mig) are selective for IL-2 activated T lymphocytes (137), while platelet factor (PF)-4 can attract monocytes and neutrophils (138). The major chemokines, their target cells and corresponding receptors are shown in the table below (Table 1.2).

Chemokine	Receptors	Target cells
C-C chemokines		
MCP-1	CCR2,CCR4 CCR9-10	Monocytes, specific lymphocyte subsets, eosinophils, basophils
MCP-2	CCR1-3 CCR9	
MCP-3	CCR1-3 CCR9-10	
MCP-4	CCR2-3 CCR9	
MIP-1α	CCR1 CCR4-5	Specific lymphocyte subsets, some monocytes, eosinophils.
MIP-1β	CCR4-5 CCR9	Monocytes, specific lymphocyte subsets
RANTES	CCR1 CCR3-5 CCR9	Specific lymphocyte subsets, some monocytes, eosinophils.
C-X-C chemokines		
IL-8	CXCR1 CXCR2	Neutrophils, basophils, monocytes
GRO-α,-β,-γ	CXCR2	Neutrophils, basophils, monocytes
IP-10	CXCR3	T lymphocytes
PF-4	CXCR3	Monocytes, neutrophils
SDF-1	CXCR4	T lymphocytes, monocytes, neutrophils

Table 1.2 Some chemokines and chemokine receptors

The effects of chemokines on leukocytes are mediated by heptahelical receptors coupled to GTP-binding proteins. Within seconds of addition of a chemoattractant to a leukocyte suspension dramatic shape changes occur. The formation and retraction of lamellipodia, which function like the arms and legs of migrating cells, is

dependent on the polymerisation and breakdown of actin. Upregulation and activation of leukocyte integrins enables their adherence to vascular endothelial cells prior to transmigration into tissues (139). Activation of the leukocyte also ensues, as evidenced by rises in intracellular calcium, production of microbicidal oxygen radicals and bioactive lipids, and release of lytic enzymes from intracellular granules (140).

In the long-established paradigm of chemotaxis, leukocytes move "up" a concentration gradient of chemoattractant. *In vivo* this concentration gradient may be formed by "diffusion barriers" such as the vascular endothelium, the vessel wall and interstitial spaces. It is becoming increasingly recognised however that cells migrating within tissues encounter multiple chemoattractant signals in complex spatial and temporal patterns. *In vitro* models have demonstrated that sequential leukocyte migration through spatially distinct attractant fields is determined by the nature and sequence of chemoattractants (141). It may be that multiple attractants participate, in a hierachical manner, to guide leukocyte subsets to their tissue destination and ensure ongoing leukocyte recruitment. This process is facilitated by each of the many chemoattractant receptors expressed on the leukocyte surface (142).

Most chemokines are produced under pathological conditions by tissue cells and infiltrating leukocytes. Some chemokines seem to fulfil housekeeping functions, however, such as leukocyte maturation within the bone marrow, the trafficking and homing of particular lymphocyte subsets within lymphoid tissue and the renewal of circulating leukocytes (143)(Fig 1.7).

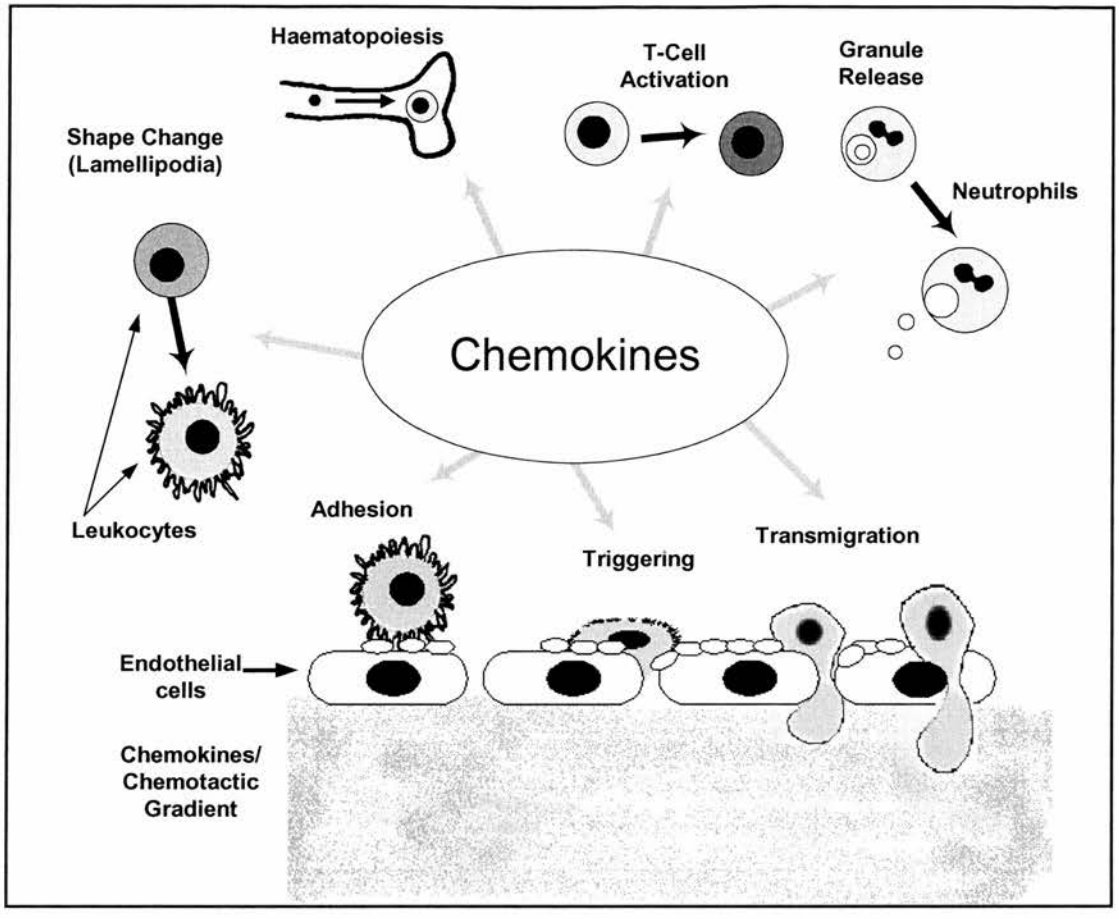


Figure 1.7

Diverse roles of chemokines in leukocyte trafficking (adapted from (143)).

Chemokines play a role in:(1) the maturation and differentiation of leukocyte subsets in the bone marrow (2) the induction of leukocyte mobility and shape changes (3) the triggering of leukocyte-endothelial adhesion and transmigration (4) generation of chemotactic gradients guiding leukocytes to the inflammatory site (5) antigen presentation and co-stimulation of activation (6) leukocyte activation.

The recently identified C-C chemokines *t*hymus and *a*ctivation *r*egulated *c*hemokine (TARC), *E*BI1-*L*igand *c*hemokine (ELC) and *s*econdary *l*ymphoid-tissue *c*hemokine (SLC) are constitutively expressed at high levels in the thymus, lymph nodes and other lymphoid tissues and attract T lymphocytes and, to a variable degree, B lymphocytes (144). The restricted, constitutive production of these chemokines in

lymphoid tissue and their apparent selectivity for lymphocyte receptors implicates their involvement in the regulation of physiological lymphocyte trafficking.

It is becoming apparent that, although originally identified as host defence proteins, chemokines clearly have other functions, for example in growth regulation, angiogenesis and tumour metastasis. PF-4 (145) and IP-10, for example, (146) inhibit neovascularisation, tumour growth and metastasis. In contrast IL-8 promotes angiogenesis and metastasis (147). Stromal-derived factor (SDF)-1 is a C-X-C chemokine which attracts T cells, monocytes and neutrophils expressing the receptor CXCR4. In addition SDF-1 attracts progenitor B cells and it has been speculated that it may be involved in directing progenitor cells into appropriate maturation sites in the bone marrow and haematopoiesis (148). Support for this theory comes from the observation that mice lacking SDF-1 have severely impaired lymphopoiesis and abnormally low numbers of B and myeloid cell bone-marrow precursors (in addition to a defective ventricular septum in the heart) (149). Mice lacking the receptor for SDF-1, CXCR4, have a similar phenotype to those lacking SDF-1, but in addition have central nervous system defects (premature and abnormal clustering of neurons) and abnormal formation of blood vessels in the gastrointestinal tract (150, 151). It has been speculated that the chemoattractant properties of chemokines may be useful during morphogenesis to keep the cells that form tissues together, but research in this field is still in its infancy and the mechanisms of these effects as yet unknown (135).

1.2.4. Chemokine receptors

The biological effects of chemokines are mediated through interactions with seven transmembrane, G-protein coupled receptors. Chemokine receptor genes are expressed in a cell-type specific manner and this may be the basis for the specificity of chemokines for leukocyte subsets (152). While most receptors bind more than one chemokine, in general CC receptors bind only C-C chemokines and CXC receptors bind only C-X-C chemokines (Table 1.2). A recent study, however, has demonstrated that binding may not be necessarily so restricted to each sub-family in that C-C chemokine 6-C-kine (also called Exodus 2) binds the CXC chemokine receptor CXCR3 (153). Five human CXC chemokine receptors (CXCR1-5) and ten human CC (CCR1-10) receptors have been identified (143). Although generally found on leukocytes, chemokine receptors can also be present on other cell types such as VSMC or endothelial cells, either constitutively or in response to inflammatory stimuli (154, 155).

Binding of a chemokine to its receptor leads to a cascade of cellular activation which may include the generation of inositol trisphosphate, the release of intracellular calcium and the activation of protein kinase C (135). Many details are still unclear and different kinases may be involved in signal transduction, including serine/threonine kinases as well as tyrosine kinases. Most studies to date have focused on IL-8 receptor signalling (156, 157). Upon IL-8 binding, receptor coupling with a *Bordetella pertussis* toxin-sensitive G protein initiates a signalling cascade which leads to activation of phospholipase C, protein kinase C, Src-related tyrosine kinases, phosphatidylinositol-3-OH kinases and protein kinase B. Phospholipase C activation, which results in the formation of inositol-1,4,5-trisphosphate, leads to

increases in intracellular calcium levels, an event which is required for granule release and superoxide production. In addition IL-8 binding results in the activation of small GTPases; these proteins regulate cytoskeletal arrangements involved in adhesion and chemotaxis such as membrane ruffling, pseudopod formation and assembly of focal adhesion complexes. Receptor binding therefore activates the intracellular pathways necessary to propel the cell towards the source of chemoattractant.

The chemokine receptors CXCR4 and CCR5 serve as co-receptors for the human immunodeficiency virus (HIV) (158). By facilitating entry into cells these receptors determine viral tropism i.e.strains of HIV-1 that infect T cells (T-tropic strains) bind to CXCR4 strains, while strains infecting macrophages and activated T-cells (M-tropic) bind to CCR5. Entry of HIV into cells is blocked by the ligands for these receptors-SDF-1, RANTES and MIP1α/β (159, 160). The importance of chemokines receptors in the pathophysiology of HIV infection is evidenced by the fact that mutations in the CCR5 receptor, if homozygous confer resistance to HIV infection and, if heterozygous, delay disease progression (161, 162).

Chemokines also interact with two types of non-signalling molecules. The erythrocyte chemokine receptor called Duffy antigen receptor for chemokines (DARC) is present on erthrocytes and endothelial cells (163). Although DARC is structurally related to the chemokine receptors, it is distinctive in that both C-C and C-X-C chemokines bind to it and ligand binding does not induce calcium flux. It has been speculated that this receptor may serve as a reservoir for chemokines, thereby serving to clear them from the circulation. Chemokines also interact with a group of

negatively charged heparin sulphate proteoglycans found in the ECM and on endothelial cells (164). It has been proposed that these interactions may serve to establish a local concentration gradient from the source of chemokine secretion.

1.3. Pathological consequences of "inappropriate" endothelial cell, vascular smooth muscle cell and pericyte activation in human disease

1.3.1. Atherosclerotic phenotype

Atherosclerosis continues to be a leading cause of death in the United States, Europe and much of Asia (165). It is now apparent that the lesions of atherosclerosis represent a series of highly specific cellular and molecular responses resulting from an intricate interplay between diverse factors such as lipid metabolism, blood coagulation, behavioural risk factors, cytokines and haemodynamic stress (166). The end result of this process is occlusive lesions affecting large and medium sized arteries resulting in ischaemia or infarction of the organ concerned, most commonly heart and brain.

The endothelial cells which line such blood vessels play a central role in both the initiation and propagation of this process. The endothelium has numerous physiological roles as discussed in detail in section 1.1.1. It provides a permeability barrier through which there is exchange and active transport of substances into the vessel wall. In addition it provides a non-thrombogenic surface and maintains the basement membrane upon which it rests. By release of vasodilators (e.g. prostaglandins, nitric oxide) or vasoconstrictors (e.g. endothelin-1) the endothelium is an important regulator of vascular tone, whilst its ability to elaborate growth regulatory factors and cytokines, although important in defence against injury, has important implications in the fibroproliferative process that characterises atherosclerosis.

It now appears that endothelial dysfunction resulting from injury (and not necessarily denudation as was previously thought), leads to compensatory changes that alter its normal homeostatic properties (reviewed in (166)). This injury can take many forms and includes such risk factors as elevated modified low density lipoprotein (LDL), hypertension, diabetes mellitus, free radicals caused by cigarette smoking and elevated homocysteine. It is proposed that endothelial injury (regardless of the initiating cause) increases endothelial permeability, as well as enhancing its adhesiveness for leukocytes and platelets. Increased expression of adhesion molecules - selectins, ICAM-1 and VCAM-1 - results in firm attachment of leukocytes to the endothelial surface while migration of leukocytes across the endothelium occurs in response to chemokines, produced by activated endothelium, smooth muscle cells and monocytes.

It is generally accepted that oxidation of LDL within the microenvironment of the arterial wall is an important early step in atherosclerosis (167). Many of the cells present in the arterial wall can oxidise LDL, including endothelial cells, vascular smooth muscle cells and macrophages (168). Transition metals (present within plaques), lipoxgenase enzymes, myeloperoxidase and reactive nitrogen species (produced *in vivo* by the reaction of nitric oxide with superoxide) have all been implicated in the oxidation of LDL within the arterial wall, an environment that is relatively depleted of aqueous phase antioxidants (169-171). A recent study has demonstrated that the 12/15 lipoxgenase enzyme in particular may play a key role in these oxidative processes (172). In this study, homozygous disruption of the 12/15 lipoxygenase gene diminished atherosclerosis in apo-E deficient mice as compared to their lipoxygenase expressing controls (without significant changes in plasma

cholesterol levels). Other non-oxidative modifications of LDL including glycation, carbamylation and modification by homocysteine, may also produce modified LDL species that share some of the properties of ox-LDL (173). The oxidation of polyunsaturated acids within LDL is followed by their fragmentation and the release of aldehydes and ketones, such as malondialdehyde and 4-hydroxynonenal, which can modify key lysine residues on apoprotein B (apoB) Modified apoB is no longer recognised by the apoB receptor, but rather is taken up by the macrophage scavenger receptors. This leads to dysregulated uptake of LDL cholesterol, the accumulation of cholesterol esters within the macrophage and the formation of the characteristic foam cell (166). The endothelium itself can also take up oxidised LDL via the recently identified lectin-like ox-LDL receptor (LOX-1) (174). *In vitro* studies have shown that, in human coronary artery endothelial cells, ox-LDL can increase its own receptor (LOX-1) expression, while *in vivo* studies have confirmed LOX-1 expression on not only endothelial cells, but also intimal smooth muscle cells and macrophages, within the atherosclerotic plaque (175, 176).

Oxidised LDL is a potent chemoattractant for circulating monocytes but inhibits their further migration after they have entered the arterial wall and differentiated into macrophages (177). In addition ox-LDL, as a result of its oxidative modification, acquires a number of important biological activities which increase its atherogenicity (166). It is cytotoxic to endothelial cells, promotes production of a wide range of cytokines and growth factors from lesional macrophages, increases platelet aggregation and interferes with the actions of nitric oxide. It can also result in the upregulation of endothelial genes such as macrophage colony stimulating factor (M-CSF) and the chemokine MCP-1. Finally, via increases in cellular adhesion

molecules, LDL has been implicated in promoting the leukocyte-endothelial cell adhesion which is central to the initiation and growth of atherosclerotic lesions.

Cell adhesion molecules have been discussed in detail in section 1.2.2. Levels of soluble cell adhesion molecules are increased in patients with dyslipidaemia and appear to be regulated by plasma cholesterol levels (178). Moreover histopathological studies have shown increased VCAM-1, ICAM-1, P- and E-selectin in atherosclerotic lesions and have defined increased adhesion molecule expression as temporally preceding intimal accumulation of macrophages and T-lymphocytes (179-182). Interestingly a recent study using mice doubly deficient in the LDL receptor and P-selectin has confirmed the importance of this adhesion molecule in the development of early atherosclerotic lesions (183). These observations are supported by *in vitro* and *in vivo* studies indicating that ox-LDL induced endothelial-leukocyte hyperadhesiveness is mediated by P-selectin, L-selectin, VCAM-1, as well as ICAM-1 and its integrin counter-receptor CD11/CD18 (184). Also, native LDL binding to the LDL receptor has been shown *in vitro* to activate human endothelial cells, as evidenced by increases in intracellular calcium which then acts as a second messenger to induce VCAM-1 and E-selectin expression (185). Generation and uptake of ox-LDL therefore helps to drive the inflammatory process by stimulating activation of macrophages and the entry of new monocytes/T lymphocytes into the arterial wall, eventually resulting in fatty streak formation, the first phase of an atherosclerotic lesion.

The elaboration of cytokines and growth factors also stimulates migration and proliferation of smooth muscle cells which become intermixed with the

inflammatory cells. As a result of this patho-immune response, progressive atherosclerotic lesions are characterised by the infiltration of immune competent cells (macrophages and T lymphocytes), the proliferation of intimal cells of the arterial wall (smooth muscle), the accumulation of lipid and the deposition of extracellular matrix components (166). Cycles of cellular accumulation, macrophage death by apoptosis and their deposition of extracellular lipid and subsequent formation of fibrous tissue leads to enlargement and restructuring of the lesion so that it becomes covered by a fibrous cap overlying a core of lipid - the so-called atherosclerotic plaque. This encroaches into the blood vessel lumen with resulting narrowing and obstruction to blood flow. Lesion complication often culminates in rupture of the fibrous cap, exposing circulating blood components to the tissue factor rich core and inciting thrombosis, as occurs during the acute coronary syndromes or myocardial infarction.

Factors affecting plaque stability have been the focus of many recent studies. A plaque prone to rupture is generally one with a thin fibrous cap overlying a large thrombogenic lipid core rich in lipid laden macrophages (186). The integrity of the fibrous cap, and thus its resistance to rupture, depends on the extracellular matrix and the balance between the synthesis and degradation of the macromolecules that constitute it (principally collagen, elastin and proteoglycans). A family of enzymes known as matrix metalloproteinases are expressed by plaque macrophages and can contribute to weakening of the fibrous cap by degradation of all its major constituents.

As with all inflammatory processes no single factor works alone in the process of atherogenesis. Complex cellular networks exist whereby the release of one molecule can lead to expression of a second molecule which itself causes gene upregulation in an autocrine or paracrine way (187). Il-1 or TNF-α for example are released by activated lesion macrophages exposed to ox-LDL. In culture these cytokines can induce PDGF gene expression in smooth muscle cells, which can in turn result in further smooth muscle cell stimulation and growth. Many such mediators have been implicated and these are illustrated schematically below. (Fig 1.8).

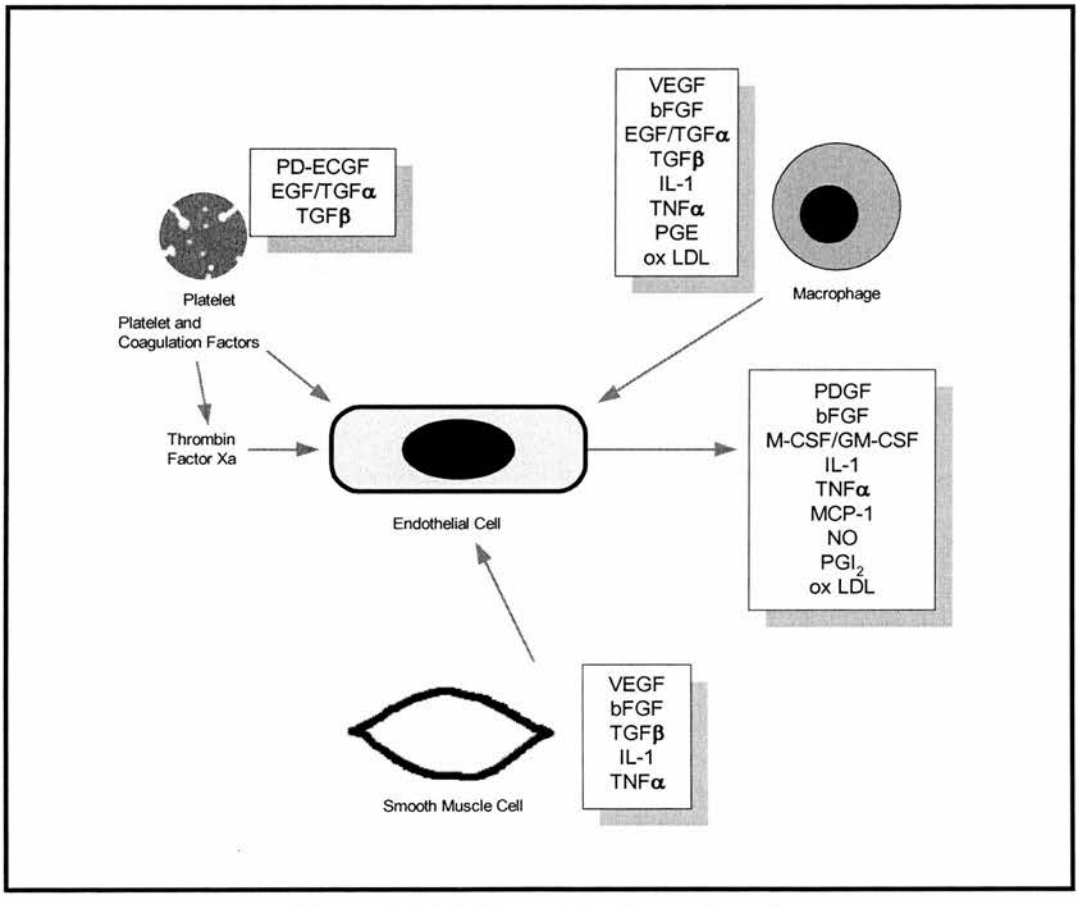


Figure 1.8 Mediators in atherosclerosis.

The principal products of platelets, macrophages and smooth muscle cells that may affect the endothelium and promote atherogenesis are shown in this schematic diagram In turn the endothelial cells can themselves express genes for and synthesise a number of growth-regulatory molecules, cytokines, chemotactic factors and vasoactive mediators (adapted from(187)).

CD40: CD40L interactions and atherosclerosis

Cells in human atherosclerotic lesions have been demonstrated to express the immune mediator CD40 and its ligand CD40L (188). CD40L is an important immunomodulatory signalling molecule previously considered to be restricted to activated $CD4^+$ T lymphocytes. Recent studies have identified its coexpression with its receptor CD40 on endothelial cells, smooth muscle cells and macrophages in the atherosclerotic plaque (188). In hyperlipidaemic mice (lacking the receptor for LDL and fed a high–cholesterol diet), inhibition of CD40 signalling by anti-CD40 antibody limited atherosclerosis. Lesions were smaller, had reduced lipid content, contained significantly fewer macrophages and lymphocytes and demonstrated reduced expression of VCAM-1. Stimulation *in vitro* of human vascular smooth muscle and endothelial cells with CD40L activates interleukin 1β-converting enzyme (ICE) activity and promotes elaboration of active interleukin 1β (189). In addition, stimulation of human monocytes/macrophages through CD40 (using either membranes of activated T cells or recombinant CD40L) has been reported to induce expression of the chemokines MCP-1 and IL-8, the pro-inflammatory cytokines IL-1 and TNF-α, tissue factor and the matrix metalloproteinases collagenase and stromelysin (190). The matrix metalloproteinase stromelysin-3 mRNA and protein has been subsequently shown to co-localise with CD40 and CD40L in atherosclerotic plaques (191). In keeping with this observation, interruption of CD40/CD40L signalling in LDL receptor deficient hyperlidaemic mice has been reported to substantially reduce expression of this enzyme in atherosclerotic plaques. These findings suggest a crucial role for CD40-CD40L T cell independent interactions in atheroma formation and the triggering of the plaque rupture that gives rise to unstable events.

Chemokines and atherosclerosis

The migration of immune cells from blood vessel lumen into the atherosclerotic plaque is central to atherogenesis. Chemokines, as discussed in section 1.2.3, are members of a superfamily of small polypeptides that mediate not only migration, but also the growth and activation of leukocytes and a variety of other cells (135). The biological effects of chemokines are mediated through interactions with seven transmembrane, G-protein coupled receptors and binding of a chemokine to its receptor activates the intracellular pathways necessary to propel the cell in its chosen direction. A number of recent investigations have implicated certain chemokines, and their receptors, in the promotion of atherogenesis (192, 193).

The expression of IL-8 by lesional macrophage-derived foam cells has been detected at various stages of the atherosclerotic process (194). Its corresponding receptor CXCR-2 has also been detected in the intima of human atherosclerotic lesions (136). In LDL-receptor deficient mice the murine homologue of this receptor (mIL-8R) was abundant in the macrophage rich areas of established atherosclerotic lesions. Moreover, the functional importance of this receptor was confirmed when its absence on bone marrow derived-peripheral blood leukocytes was associated with reduced lesion size and reduced macrophage recruitment, implying a role for its cognate ligands (IL-8, growth-related oncogene (GRO) and neutrophil activating protein-2 [NAP-2]) in the localisation of macrophages to these lesions (136). Il-8 and its related chemokines were originally thought to be predominantly chemotactic for neutrophils which are scanty in human atherosclerotic lesions. The increasing evidence indicating a role for this chemokine in monocyte/macrophage recruitment to the atherosclerotic plaque, together with the recent *in vitro* finding that IL-8 is a

potent trigger for firm adhesion of monocytes to vascular endothelium expressing cell adhesion molecules under flow conditions, strongly suggests that this chemokine is also a potent chemoattractant for mononuclear cells (195).

Expression of the CC chemokine MCP-1 is also upregulated in human atherosclerotic plaques (196). Studies *in vitro* have demonstrated MCP-1 upregulation in endothelial cells, smooth muscle cells and monocytes in response to various stimuli known to be present in lesions (192). These include oxLDL, minimally modified LDL, the pro-inflammatory cytokines IL-1 and TNF-α, the growth factors PDGF and basic FGF and binding of CD 40 ligand to its receptor CD 40. MCP-1 modulates not only monocyte (and T lymphocyte) recruitment but also monocyte activation, including expression of some complement proteins, IL-1 and the procoagulant tissue factor. In atherosclerotic prone mice lacking the receptor for MCP-1, CCR2, there was markedly decreased lesion formation and macrophage recruitment without changes in cholesterol levels or distribution of lipoprotein particles (197). This identified CCR2 as a possible genetic determinant of murine atherosclerosis and provided evidence for the role of MCP-1 in macrophage recruitment and atherogenesis. Further confirmation of this was achieved when deletion of the MCP-1 gene in transgenic mice expressing human apolipoprotein B (causing elevated plasma cholesterol levels) provided protection from macrophage recruitment and lesion formation, without altering lipoprotein metabolism (198). Conversely, when bone marrow cells from mice overexpressing a murine MCP-1 transgene were transplanted into irradiated apolipoprotein E-knockout mice, macrophages expressing the transgene were found in several tissues including the aorta (199). Atherosclerotic lesions in these mice showed increased oxidised lipid

content and macrophage infiltration providing further evidence that MCP-1 expression by leukocytes, mainly macrophages, may increase the progression of atherosclerosis by facilitating both macrophage infiltration and oxidised lipid accumulation.

Platelet factor 4 (PF 4) and interferon-inducible protein-10kDa (IP-10) are C-X-C chemokines which bind to the chemokine receptor, CXCR3. PF-4 selectively binds to regions of active endothelial cell migration (and angiogenesis) and inhibits endothelial cell proliferation (192). It also inhibits thrombin-induced matrix metalloproteinase MMP-1 and MMP-3 expression by endothelial cells *in vitro* but does not affect TIMP expression. It has been speculated therefore that PF4 may interfere with vascular wall remodelling in atherosclerosis. IP-10, like PF-4, inhibits endothelial proliferation. IP-10 mRNA is induced in the rat carotid artery after balloon angioplasty (200). The capacity of IP-10 to modulate arterial remodelling is further supported by its expression in VSMC *in vitro* and its chemotactic and mitogenic actions on these cells (200).

Other C-C chemokines which have been demonstrated in atherosclerotic plaques are *p*ulmonary and *a*ctivation *r*egulated *c*hemokine (PARC), ELC, RANTES and MIP-1α (201). While PARC was restricted to macrophages, ELC is present on both macrophages and smooth muscle cells. Furthermore ELC mRNA levels *in vitro* have been demonstrated to be upregulated in aortic smooth muscle cells stimulated with TNF-α and IFN-γ. These chemokines have a highly selective activity on lymphocytes leading to speculation that they may be involved in the recruitment of T cells into the atherosclerotic plaque. Although RANTES mRNA has been detected in

atherosclerotic tissue (202), its presence also in normal aortic tissue makes the significance of this finding unclear (154).

Chemokine receptors and atherosclerosis

Chemokine receptors have also been demonstrated on the various cells present in the atherosclerotic lesion. Chemokine receptor mRNA for CCR-1 and CCR-2 have been demonstrated on human vascular smooth muscle cells (154). Endothelial cells express CXCR-4 (155) but conflicting reports exist as to the presence of CCR1, CCR2 and CXCR2 on these cells (155, 203-205). The constitutive nature of such receptors and their chemokines (stromal derived factor(SDF)-1 in the case of CXCR-4) may be important in the early recruitment of monocytes and T lymphocytes as opposed to the inducible chemokines such as MCP-1 which are generally not expressed on normal arteries. Moreover, the expression of these receptors can be regulated by pro-inflammatory cytokines (155). Further studies will help to clarify the role of these chemokines and chemokine receptors in atherogenesis.

1.3.2. Vasculitic phenotype

Vasculitis is defined simply as blood vessel inflammation. A broad and heterogeneous group of syndromes result from this process, since any type, size and location of blood vessel may be involved. Patients typically present with symptoms related to inflammation/ischaemia in the particular vascular bed affected; disease may be confined to a single organ or it may simultaneously involve several organ systems (206).

Classification of the vasculitic syndromes has been plagued by their variability and overlap, but in general relies principally on the size of the primary blood vessel affected (Table 1.3).

Evidence for the immunopathogenenic mechanisms underlying vasculitic disease is for the most part indirect and may reflect epiphenomena as opposed to true causality. It is generally held, however, that an initiating stimulus leads to vascular injury (often segmental or localised), resulting in inappropriate activation of the normally tightly regulated immune response and subsequent vessel damage. Putative stimuli include immune complex deposition in response to exogenous antigens, complement activation, infectious agents and anti-neutrophil cytoplasmic antibodies (ANCA)/ anti-endothelial cell antibodies (AECA).

Classification of Vasculitic Syndromes	
Clinical Syndrome	**Predominant Vessels Affected**
Takayasu arteritis	Large arteries (aorta & primary branches)
Giant cell (temporal) arteritis	Large and medium arteries (aorta, primary, and secondary branches)
Systemic necrotizing vasculitis	Medium and small muscular arteries and arterioles (diverse distributions and locations)
Systemic necrotizing vasculitis	
Polyangiitis nodosa	
Allergic granulomatosis and angiitis (Churg-Strauss syndrome)	
Overlap syndrome	
Wegeners granulomatosis	
Vasculitis in rheumatic disease (eg, rheumatoid arthritis. Behcets syndrome)	
Granulomatous angiitis of central nervous system	
Kawasaki disease	
Microscopic polvangiitis	
Henoch-Schönlein purpura	
Mixed cryoglobulinemia	
Hypocomplementemic urticarial vasculitis	
Vasculitis associated with malignancy	
Cutaneous vasculitis associated with other diseases (eg, biliary cirrhosis, ulcerative colitis)	
Hypersensitivity vasculitis (cutaneous leukocytoclastic vasculitis)	Small vessels (arteries capillaries, venules)
Large vessel refers to the aorta and the largest branches directed toward major body regions (eg, to the extremities and the head and neck): medium vessel refers to the main visceral arteries (eg. renal, hepatic, coronary, and mesenteric arteries): and small vessel refers to venules, capillaries, arterioles, and the intraparenchymal distal arterial radicals that connect with arterioles. Some small and large vessel vasculitis may involve medium arteries, but large and medium vessel vasculitis do not involve vessels smaller than arteries. (206)	

Table 1.3 Classification of Vasculitic Syndromes

It may be that in certain forms of vasculitis, some form of persistant immune challenge or chronic antigen exposure induces the formation of immune complexes in excess of the capacity of normal immune system clearance mechanisms (207). Such complexes, when formed in excess, are deposited in vessel walls where they stimulate complement activation. C5a generation, for example, results in neutrophil recruitment to the site of immune complex deposition; basophil/mast cell degranulation also occurs with subsequent release of vasoactive amines and associated increases in vascular permeability. Neutrophils, activated as they try to phagocytose immune complexes, release inflammatory mediators and proteolytic enzymes. This results in endothelial damage, exposure of the sub-endothelium and activation of the intrinsic coagulation pathway. In addition, platelets bind at sites of endothelial damage, interact with immune complexes and aggregate at these sites. Uncontrolled these events lead to the formation of microthrombi, vascular occlusion and necrosis.

Antibodies recognising antigens in the cytoplasm of ethanol fixed neutrophils (ANCA) have been detected in various forms of vasculitis and have been classified according to their pattern of immunofluorescent staining (208). Cytoplasmic anti-neutrophil antibodies (c-ANCA) bind to the enzyme proteinase-3 and stain leukocytes in a granular cytoplasmic manner. Conversely, perinuclear anti-neutrophil antibodies (p-ANCA) generally recognise the enzyme myeloperoxidase (less commonly recognised antigens include elastase, cathepsin G, lactoferrin and lysozyme) and show perinuclear staining of leukocytes. c-ANCA has a high specificity for Wegener's granulomatosis (WG) with antibody level fluctuating with disease activity; p-ANCA is most frequently detected in patients with microscopic

polyangiitis. *In vitro* c-ANCA increases the expression of E-selectin and VCAM-1 on endothelial cells, as well as E-selectin-dependent neutrophil adhesion (208). In addition, the proinflammatory cytokines TNF-α, IL-1β and IFN-γ result in the translocation of cytosolic proteinase-3 to the cell membrane of endothelial cells *in vitro* (209). Subsequent stimulation of such cytokine-primed cells with c-ANCA results in a dose-dependent induction of the phosphoinositide hydrolysis signal transduction pathway, concomitant with an increase in trans-endothelial protein leakage and loss of barrier function (209). ANCA have also been reported to stimulate neutrophil activation and degranulation and it has now been established that, at least *in vitro,* the Fc region of ANCA interacts with Fc receptors on neutrophils (210). Neutrophils constitutively express the Fc receptors FcγRIIa (CD32) and FcγRIIIb (CD16); c-ANCA and p-ANCA can bind to both these receptors resulting in neutrophil activation, degranulation and increases in adhesion molecule (CD11b/CD18) expression. Taken together, the above studies suggest that these antibodies, acting via multiple mechanisms, may be important in the pathogenesis of the vasculitic process in patients in whom they are detected.

Patients with systemic autoimmune disease associated with vasculitis, and some primary vasculitic processes (e.g. Takayasu's arteritis) have a high prevalence of anti-endothelial cell antibodies (AECA), the titre of which has been shown to correlate with disease activity (211). These antibodies, frequently associated with anionic phospholipids, activate endothelial cells *in vitro*, leading to adhesion molecule expression and, in some cases, induction of endothelial apoptosis (212, 213). In Kawasaki vasculitis, IgM anti-endothelial antibodies directed against endothelial surface antigens inducible by cytokines have been found. Thus, in some

forms of vasculitis, inducible antigens may lead to a subsequent antibody response which can lead to vascular injury.

Hepatitis B antigen, streptococcal, staphylococcal and mycobacterial antigens have been identified in certain vasculitic lesions, most commonly polyarteritis nodosa (PAN). In selected cases of PAN circulating immune complexes, containing hepatitis B surface antigen linked to specific IgG and IgM, have been demonstrated both in the circulation and in vasculitic lesions (206). Several viral infections in humans are also capable of causing direct cytopathic injury to arterial endothelium (214). Interest has also recently focused on bacterial superantigens which, by virtue of their structure, have the ability to stimulate a large portion of the T cell pool. It has been speculated that such antigens may stimulate T cells, autoreactive to some vascular wall component, to mediate autoimmune vessel wall destruction (215). Furthermore such antigens may activate autoreactive B cells to produce auto-antibodies such as ANCA, or anti-endothelial cell antibodies or indeed others, as yet unidentified.

Molecular studies in giant cell arteritis indicate that T cells are recruited to the vessel wall and are activated locally; subsequent production of IL-2 and IFN-γ regulates the activity of tissue infiltrating macrophages (216). Growth factors are instrumental in the process of intimal hyperplasia, leading to lumen obstruction and tissue ischaemia. The levels of IL-2, IFN-γ and PDGF in vascular lesions varies according to the clinical manifestations of the disease e.g. ischaemic optic neuritis or stroke is characterised by high IFN-γ and PDGF, while dominant IL-2 production is associated with polymyalgia rheumatica. It has been speculated that these different inflammatory pathways may reflect differences in the contribution of the arterial

wall, or may be due to the presence of multiple disease-inducing antigens with different tissue distributions or tropisms (216).

The prognosis of Henoch-Schonlein purpura (HSP), unlike some of the other forms of vasculitis, is excellent, with most patients recovering completely, and in some cases without the need for therapy (207). The underlying pathogenesis is presumed to be immune-complex deposition and a number of inciting antigens have been suggested including upper respiratory tract infections, various drugs, foods, insect bites and immunisations. IgA is most commonly found in the immune complexes, and this in addition to alterations in circulating serum terminal complement complexes (decreased properdin, increased C3d), suggests IgA-mediated alternate pathway complement activation. Whether the IgA complexes trigger this complement activation is still unclear. IgG anti-mesangial cell antibodies have also been detected in patients with HSP where levels have been reported to parallel the course of the renal disease (217).

It is important to note, however, that to date in most cases of vasculitis, definitive identification of causative agents has not yet been made. It is also unclear why certain individuals develop vasculitis in response to antigenic stimuli, whereas others do not. It is likely that a number of factors are involved in the ultimate expression of a vasculitic syndrome such as genetic predisposition and the regulatory mechanisms associated with the immune response to certain antigens. In addition, the size and physicochemical properties of immune complexes (if present), the relative degree of turbulence in blood flow, the haemostatic pressure in different vessels and the pre-existing integrity of the endothelium may all play a role in determining why only

certain immune complexes cause vasculitis and why vasculitis is selective for only certain vessels in individual patients.

1.3.3. Thrombotic phenotype

Blood coagulation is mediated by cellular components and soluble plasma proteins (reviewed in (218)). In response to vascular injury, circulating platelets adhere, aggregate, and provide cell-surface phospholipid for assembly of blood-clotting enzyme complexes. A series of enzymatic reactions then leads to the conversion of soluble fibrinogen to a fibrin clot (219)(Fig 1.9).

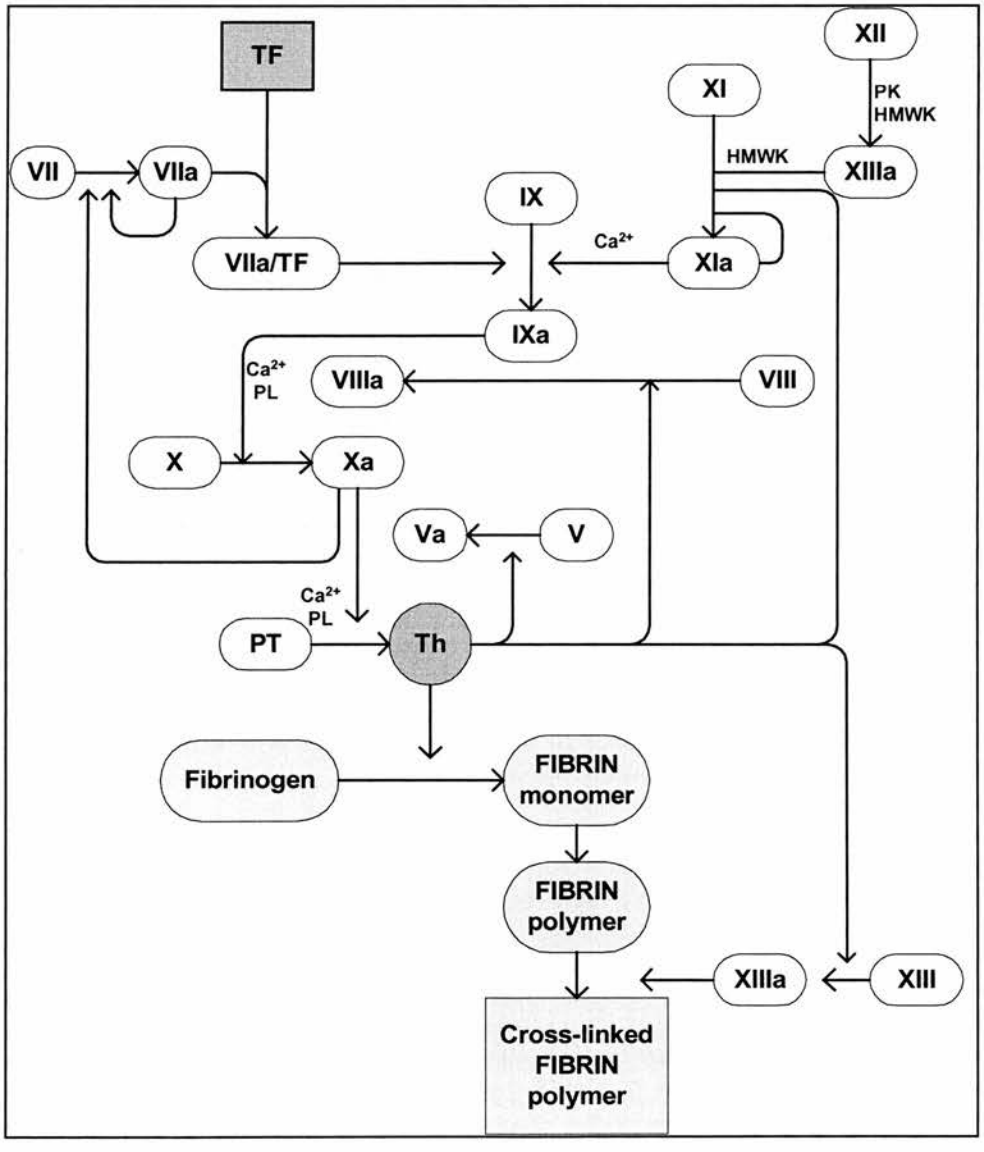


Figure 1.9 Coagulation cascade (adapted from 219) refer to following text for details

HMWK=high molecular weight kinogen: PK=prekallikrein: PL=phospholipid: PT=prothrombin: TF =tissue factor: Th=thrombin

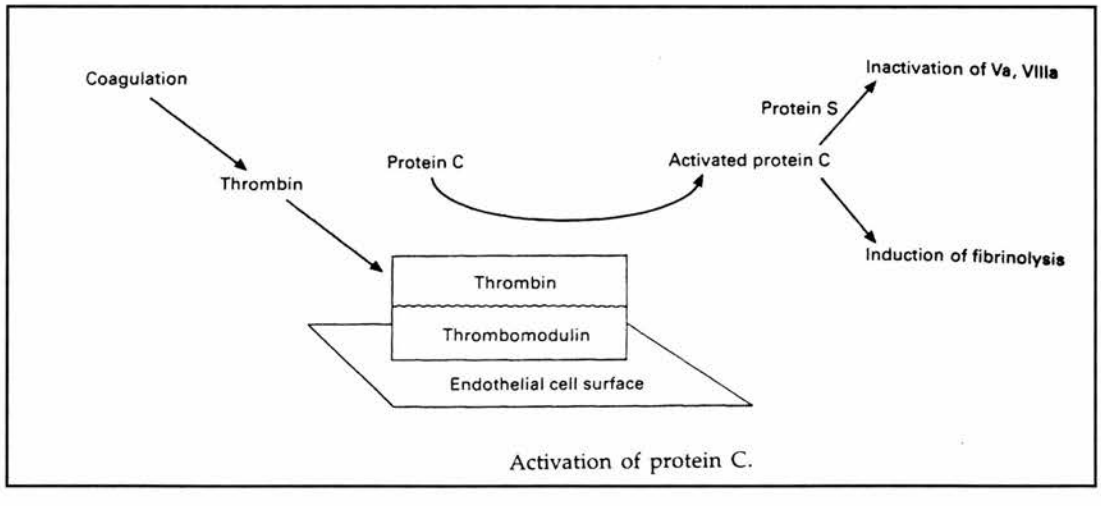
All of the enzymes (factors) within the pathways (except factor VIII) are serine proteases and hydrolyse peptide bonds. In the extrinsic pathway, tissue factors (lipoproteins released from damaged cells) bind to activated factor VII and the resulting enzyme complex activates factors IX and X of the intrinsic and common pathways respectively. Factor IX in turn activates additional factor X in a reaction accelerated by a cofactor, factor VIII. Factor VIII is a complex protein made up of a small protein molecule (VIII:C) with coagulant activity and a larger protein molecule (VIIIR:Ag) which is associated with platelet function, particularly their adhesion to exposed collagen. (Von Willebrand's factor is part of the VIIIR:Ag molecule).

Once activated, factor X converts prothrombin to thrombin (in a reaction catalysed by factor V), and thrombin in turn cleaves fibrinogen to form fibrin monomers. Polymerisation and linking of fibrin (via factor XIII) then occurs to form a chemically stable clot. Feed-back loops exist whereby thrombin itself can activate cofactors V and VIII resulting in amplification of this cascade. In essence then, the extrinsic pathway initiates coagulation, while the intrinsic pathway amplifies the response through feed-back mechanisms.

Platelets have a number of functions in, and are crucial to, thrombus formation (105, 218). Adherence of platelets to the subendothelial matrix, exposed by vascular injury, results in their activation which in turn causes recruitment and adherance of further platelets – platelet aggregation. Fibrinogen and von Willebrand factor form crossbridges between adjacent glycoprotein (GpIIb/IIIa) molecules on adjacent activated platelets thereby resulting in this platelet aggregation which in itself can form a haemostatic plug. This process is enhanced by platelet production of ADP and

thromboxane A_2 (TxA_2). TxA_2 is also a potent vasoconstrictor resulting in the retention of both platelets and clotting factors at the site of endothelial injury. Prostacyclin (PGI_2), synthesised by the vascular endothelial cell, promotes vasodilatation and inhibits platelet aggregation. PGI_2 production is also stimulated by thrombin and may play a role in limiting coagulation to the site of damage.

These cascades have the ability to transduce a small initiating stimulus/injury into a large fibrin clot and their potentially explosive nature is offset by natural anticoagulant mecanisms. Antithrombin III is a plasma protein that inhibits the activity of the serine-proteases of the intrinsic and common pathways by forming stable complexes with them. Heparin sulphate, released from endothelial cells, binds to antithrombin III and greatly potentiates its ability to inactivate these enzymes (58). Thrombin complexes with the endothelial cell via a receptor protein, thrombomodulin (59). This increases the generation of activated protein C which cleaves activated factors V and VIII and destroys their coagulant properties (60). This reaction can also be accelerated by a cofactor, protein S (Fig 1.10).



Activation of protein C.

Figure 1.10 (220)

Inhibition of the extrinsic pathway occurs via tissue factor pathway inhibitor which is a lipoprotein-associated plasma protein that forms a quaternary complex with tissue factor and factors VII and X, thereby preventing their activity. Finally the generation of plasmin from plasminogen, via another series of linked enzymatic reactions, results in the dissolution of pre-formed clots by fibrin breakdown (Fig 1.5)

The mechanisms that underlie the thrombotic phenotype are defined by Virchow's triad: a decrease in blood flow (the deep veins of the leg, for example, are particularly at risk of thrombosis in conditions of decreased flow/stasis such as the peri- and post-operative period), injury to the vessel wall and a change in the systemic balance of procoagulant and anticoagulant factors. It is interesting, however, that *systemic* changes in haemostatic mechanisms, whether congenital or acquired, typically give rise to *local* thrombotic lesions in discrete segments of the vascular tree, leading to speculation that the endothelium integrates different extracellular signals and responses according to its location within the vasculature (218).

Hypercoagulable states

Congenital deficiencies of antithrombin III, protein C and protein S are associated with an increased risk of lower limb deep-vein thrombosis (221). Resistance to activated protein C (APC) is the most common inherited hypercoagulable state found to be associated with venous thrombosis. It is caused by a single point mutation in the factor V gene. The occurrence of this mutation results in loss of one of the three factor V cleavage sites for APC and hence resistance to its anticoagulant effects. This so-called Factor V Leiden is associated with a 5-10 fold increased risk of deep

venous thrombosis in the legs, as well as an increased risk of cerebral thrombosis. Most studies have not confirmed an association between factor V Leiden and arterial thrombosis, but it may confer an increased risk of myocardial infarction in young female smokers (222). The second most common inherited risk factor for thrombosis is a point mutation in the prothrombin gene (G20210A) which is associated with increased prothrombin activity (221). This also predisposes to venous thrombi in legs and brain and may be a genetic risk for coronary and cerebral arterial thrombosis, although this needs further confirmation (223, 224).

Inherited abnormalities of the coagulation system such as those described above may interact with environmental/acquired factors to potentiate the risk of thrombosis. Trauma, surgery, immobilisation, pregnancy, the puerperium and the use of oral contraceptives are important risk factors for venous thrombosis. Oral contraceptive use, for example, results in alterations in the concentrations of many haemostatic and fibrinolytic components of the blood. In patients with the factor V Leiden mutation and prothrombin G20210A mutation, the risk of cerebral and lower limb venous thrombosis is significantly increased by concomitant oral contraceptive use (225).

Several acquired hypercoagulable states also occur. Paroxsymal haemoglobinuria is a rare condition in which an abnormal stem cell clone leads to a proportion of red cells which are sensitive to activated complement, resulting in intravascular haemolysis (218). In addition, the production of abnormal platelets leads to a tendency to thrombosis, but interestingly, this most commonly affects the hepatic, portal and mesenteric veins. In polycythaemia and myelofibrosis elevated levels of abnormal platelets which have an increased tendency to aggregate, predispose to both arterial

and venous thrombi (218). In the anti-phospholipid antibody syndrome (APAS) there is a propensity to form clots within particular venous and arterial segments of the arterial tree including retina and placenta. The mechanisms of thrombus formation by these antibodies is not completely understood, although *in vitro* they can inhibit protein C activation and activate endothelial cells (226). Furthermore in a mouse model of microcirculation, antibodies introduced from patients with APAS results in enhanced thrombus formation (227).

Haemolytic uraemic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP) are microvascular syndromes in which microangiopathic haemolytic anaemia, thrombocyopaenia and platelet clumping lead to microvascular thrombosis in all organs except liver and lungs (228). HUS is mostly due to verotoxin producing E. Coli (E.Coli 0157:H7), while the cause of TTP is still uncertain although associations with malignancy, bone marrow transplantation and HIV infection have been documented (229). Endothelial cell injury and intravascular platelet aggregation have been implicated in the pathogenesis of both syndromes. Immunohistochemical studies have demonstrated the presence of abundant von Willebrand factor in the thrombotic lesions and, particularly in the chronic relapsing forms of these syndromes, unusually large circulating multimers of von Willebrand factor occur which are capable of agglutinating platelets (230). It has been speculated that systemic endothelial injury may lead to release of excessive amounts of these abnormal multimers which, under normal circumstances, are processed to smaller forms by von Willebrand factor cleaving protease. Recent studies have indicated that, in the case of TTP, familial forms are associated with a constitutional deficiency of this enzyme, whereas in the non-familial forms the presence of an antibody inhibitor

to the protease can be demonstrated (230, 231). These abnormalities were not detected in HUS and the pathogenesis of this disease remains unclear.

The acute inflammatory response in meningococcaemia leads to activation of the cytokine and complement networks, in addition to activation of the homeostatic cascades of procoagulation, anti-coagulation and fibrinolysis (232). This disseminated coagulopathy results in an acquired deficiency of the anti-coagulants protein C, protein S and antithrombin III. Protein C activity is reduced to a greater extent than the other anticoagulants and there is a correlation between the severity of the acquired protein C deficiency, the extent of thrombotic skin lesions and adverse outcome (233). Mortality in this disease is greater than 50%, and in survivors skin grafts, amputation and end-organ failure are not uncommon. A recent study showed a decrease in mortality and morbidity in response to early protein C replacement (in additional to conventional therapy) in patients with meningococcal septicaemia and associated disseminated coagulopathy (232). Study numbers however were small and additional randomised controlled trials are necessary to confirm these preliminary results.

Elevated plasma homocysteine levels are typically caused by genetic defects in the enzymes involved in homocysteine metabolism or nutritional deficiencies in vitamin co-factors (folate, vitamin B12 and vitamin B6) (reviewed in (234)). Elevated levels also occur in chronic renal failure, in association with certain malignancies, and occasionally secondary to drugs (methotrexate, phenytoin, theophylline). In homocysteinuria (homozygous for cystathionine β-synthase deficiency) where there is marked elevations of plasma homocysteine levels, there is, in addition to skeletal

abnormalities and mental retardation, severe premature atherosclerosis and increased incidence of arterial and venous thrombosis (234). Multiple studies have also confirmed that moderately elevated homocysteine levels are an independent risk factor for atherothrombotic disease and venous thrombosis. The precise molecular mechanism underlying this is unknown, but homocysteine does cause endothelial dysfunction at several levels. In relation to coagulation, homocysteine enhances the activity of factor V, induces the expression of tissue factor, depresses the activation of protein C, inhibits the expression of thrombomodulin, and suppresses endothelial production of heparin sulphate. In aggregate these effects facilitate the production of thrombin and create a prothrombotic environment.

Vascular bed specific hyper-coagulable states

In all of these situations, a systemic defect in the haemostatic pathway, caused by absence of a circulating endogenous anticoagulant or the presence of an activated cell surface, results in an increased risk of localised thrombosis. The endothelium within a given area of the vasculature, and the microenvironment surrounding it, is crucial to maintaining the balance of pro-coagulant and anti-coagulant forces. In this scenario, not only does the microenvironment vary from one vascular bed to another in terms of the array of extracellular signals regulating the haemostatic properties of the endothelium, but there is also increasing evidence to suggest that endothelial cells from different vascular beds respond differently to the same signals in terms of their pro- and anti-coagulant activity (218).

Microenvironment: extra-cellular mediators, haemodynamic forces, cell-cell interactions

Growth factors and cytokines, constitutive to the microenvironment surrounding an endothelial cell, or produced by infiltrating cells in response to injurious stimuli, are important in defining the balance between a pro-thrombotic and anti-thrombotic phenotype. IL-1, TNF-α, and TGF-β all decrease the expression of thrombomodulin *in vitro* (235) and, in addition, the latter two cytokines increase expression of the plasminogen activator inhibitor type 1 (PAI-1) (236). Vascular endothelial growth factor (VEGF) increases the expression of thrombomodulin (237), PAI-1 and tissue type plasminogen activator (238), while PDGF increases expression of von Willebrand factor (part of the factor VIII complex) (239). These effects obviously have potential to shift towards the thrombotic phenotype, although the effects *in vivo* have yet to be clarified.

Haemodynamic forces may also be important regulators of the thrombotic phenotype. These forces will be discussed in more detail in chapter 2; suffice to say here that such forces, consisting of both shear stress - as blood flows along the endothelial surface - and cyclic strain - elongation of the cells as they undergo the rhythmic distension of the vessel, - are higher in arteries than veins. Shear stress is accentuated at sites just distal to bifurctations in vessels, cyclic strain is increased in hypertension and both forces increase during cardiac systole and exercise. Many *in vitro* studies have documented shear stress-dependent induction of endothelial genes including thrombomodulin, tissue plasminogen inactivator, tissue factor and ecNOS (218). Similarly, cyclic strain upregulates endothelial transcription of the genes for tissue plasminogen activator and inactivator and ecNOS (240). In view of these

findings, it has been speculated that changes in haemodynamic forces may contribute to the different patterns of expression of pro- and anti- coagulant factors in different vascular beds.

Communication between different cell types may also be important in this process. *In vitro,* for example, expression of PAI-1 in endothelial cells is down-regulated by conditioned medium from pulmonary artery smooth muscle cells, but up-regulated by smooth muscle cells from aorta and umbilical vein (241, 242). In another study the expression of von Willebrand factor in the endothelial cells of the heart was shown to be induced by a cardiomyocyte-dependent pathway (243).

Endothelial cell responsiveness: origin-dependent

An important component to differential endothelial responses to the extra-cellular stimuli within their microenvironment is the uneven distribution of the various pro- and anti- coagulant factors throughout the vascular tree. Levels of von Willebrand factor, for example, vary from one vascular bed to another, while immunohistochemstry has revealed high levels of the thrombomodulin receptor in the endothelium of the lungs and heart, with barely detectable levels within the blood-brain barrier (218). Gene targeting studies in mice have provided proof of this concept in that mice deficient in either thrombomodulin or tissue-type plasminogen activator have characteristic patterns of thrombus distribution, with some organs being spared (244).

The response of a given endothelial cell to a stimulus also varies. *In vitro,* for example, plasma derived from patients with TTP mediates differential effects on the

production of prostacyclin and apoptosis in endothelial cells derived from various vascular beds (245). IL-1α inhibits expression of VCAM-1 in dermal microvascular endothelial cells but not other endothelial cell types where it induces expression of this molecule (246). Furthermore, stimulation of blood vessels *ex vivo* with thrombin has been shown to mediate an endothelial-dependent relaxation of the internal thoracic artery but causes contraction of the saphenous vein (247). The expression of von Willebrand factor within the endothelial microvessels of the heart is governed by a signalling pathway mediated by cardiomyocyte dependent PDGF AB heterodimer. PDGF AB is not detectible in other vascular beds such as lung, and even within the cardiac microvasculature, only certain endothelial cells have receptors for PDGF-α and thus are able to transduce the signal (239). Modulation of tissue factor activity by the pro-inflammatory cytokine, TNF-α varies *in vitro* according to the origin of the endothelial cell, be it aorta, umbilical vein or dermal microvessels (218).

A final mechanism underlying the expression of vascular-bed-specific phenotypes may be found at the level of transcription, with endothelial genes being controlled by different pathways. In transgenic mice for example, a short 733-bp region of the von Willebrand promoter directs expression only in cerebral endothelium, whereas a promoter containing additional upstream and downstream elements directs expression in the endothelium of heart and skeletal muscle (243, 248).

In summary then, regional networks of pro and anti-coagulant mechanisms yield a net balance in hemostasis. In hypercoagulable states, abnormalities of the coagulation factors, either congenital or acquired and often interacting with environmental factors, predispose to thrombosis. The endothelium plays a crucial

role in the maintenance of haemostasis and it is now apparent that endothelial cells from different vascular beds respond differently to the same signals in terms of their pro- and anti-coagulant activity. This, in addition to the various extra-cellular mediators, haemodynamic forces and cell-cell interactions occuring in the microenvironment within a given vascular bed, explains why systemic defects in the haemostatic pathways generally result in localised thrombosis.

1.3.4. Vasoconstrictive phenotype

A variety of human diseases are characterised by vasoconstriction e.g. ischaemic acute tubular necrosis, Raynaud's phenomonen, sclerodermic crisis and pre-eclampsia. In these diseases diverse stimuli, through injury to endothelium, jeopardise the endothelial-dependent control of vascular tone. In addition, as has been extensively discussed, endothelial activation-injury promotes the adaptation of a pro-inflammatory phenotype by these cells (249-251). For the purposes of this discussion, pre-eclampsia will be used to illustrate the characteristics of the vasocontrictive phenotype.

Pre-eclampsia refers to the development of hypertension and proteinuria during the second and third trimesters of pregnancy. This condition is most common in, but not exclusive to, primigravidae and occurs in about 1% of this group. In addition to increased foetal risks of intrauterine growth retardation or intrauterine death, it poses significant threat to the mother as it may precede eclampsia. Eclampsia is characterised by convulsions and coma and carries increased risks of maternal death from cerebral ischaemia/haemorrhage, renal, hepatic and cardiac failure. While the pathogenesis of this disease remains poorly understood, there is now abundant circumstantial evidence supporting the concept of endothelial dysfunction and activation in this multisystem disorder, in addition to a systemic activation of maternal inflammatory responses (249). Indeed most clinical aspects of pre-eclampsia could potentially be explained by generalised maternal endothelial cell dysfunction: intense vasoconstriction and hypertension through disturbed endothelial control of vascular tone, fluid retention resulting from increased endothelial

permeability and clotting dysfunction due to abnormal endothelial procoagulant production.

It has been speculated that the pathogenesis of pre-eclampsia is related to an imbalance of oxidative homeostasis. Maternal dyslipidaemia, which may be in part related to fatty acid mobilisation for the increased energy demands of pregnancy, and altered iron kinetics may both contribute to the increased oxidative load (252). Increased levels of lipid peroxides have been detected both in the placenta and in the circulation of pre-eclamptics and these can result in activation of maternal endothelial cells and circulating leukocytes (253). Endothelial activation results in upregulation of adhesion molecules; a concurrent upregulation of the corresponding receptors on neutrophils results in increased neutrophil-endothelial binding and transmigration (254). Upon activation neutrophil granules are released, leukotrienes are synthesised and superoxide is generated in a respiratory burst. In addition, complement activation and increased monocyte production of the cytokines TNF-α, IL-6 and IL-8 have also been demonstrated (255). These processes both promote and sustain vascular damage. Loss of endothelial integrity and increases in vascular permeability can be demonstrated in patients who have increased circulating levels of the endothelial cell markers fibronectin, von Willebrand factor, tissue plasminogen activator and plasminogen activator inhibitor-1 (PAI-1) (256). Increases in levels of endothelin-1 may also contribute to the intense vasoconstriction and hypertension that characterises this disease (257).

Placental tissue is essential to to generate the end stages of the syndrome of pre-eclampsia. Furthermore, in the rare case of extrauterine pregnancy where delivery of

the foetus does not lead to delivery of the placenta, signs of the disease persist post-partum. It has been speculated that defective placentation may cause uteroplacental arterial insufficiency and subsequent placental hypoxia (249). This may result in release of cytokines, proteolytic enzymes and free radicals into the maternal circulation leading to endothelial and leukocyte activation as described previously. Increasing placental damage also results in release of syncytiotrophoblastic microvillus fragments which may themselves initiate an inflammatory response.

Pregnancy has long been regarded as a state of relative immunosuppression, but some interesting recent observations have suggested that normal pregnancy is also associated with a degree of immune activation. Using flow cytometric techniques, leukocytes from women with normal pregnancies showed higher surface expression of the integrin CD11b and the IgG receptor CD64 as compared with non-pregnant women (256). Intracellular reactive oxygen species were also significantly increased, though not to the degrees observed in pre-eclampsia. This has led these authors to suggest that a generalised, intravascular, inflammatory response occurs in normal pregnancy and that pre-eclampsia is not an intrinsically different state of pregnancy, but rather represents the extreme end of a universal maternal response to pregnancy. Decompensation might result when the inflammatory stimulus is too strong (e.g. placental hypoxia) or the maternal response to it is too intense (e.g. genetic susceptibility to inflammatory stimuli). These are complex issues and important questions remain unanswered such as the preponderance of pre-eclampsia in first pregnancies and the occurrence of marked vasoconstriction/hypertension in this disease. Acute sepsis for example, the classical paradigm of systemic inflammation,

is classically associated with the opposite clinical syndrome i.e. vasodilatation and hypotension.

1.3.5 Alloreactive phenotype

Allograft Rejection: Major histocompatibility complex

The ability to distinguish self from non-self is the basis for the rejection process. Allograft rejection, both acute and chronic, represents the end result of a complex set of immunological processes arising from the recognition of non-self antigens (peptides) on transplanted tissues (258). Major histocompatibility complex molecules (MHC) are the most potent of the histocompatibility antigens that serve as targets for allograft rejection. The MHC, first described in the mouse by Gorer (259) and Snell (260), is a closely linked series of genes encoding the major antigens responsible for organ rejection in all mammalian species studied to date. In humans this polymorphic set of membrane proteins are known as the human leukocyte antigens (HLA), homologous to RT1 in the rat and H-2 in the mouse (Table 1.4).

MHC Antigens			
		Class I	Class II
Human	HLA	ABC	DQ DR DP
Rat	RT1	A	B D
Mouse	H-2	KDL	I-A I-E

Table 1.4 MHC antigens

MHC antigens are divided into two groups: class I and class II. Class I molecules are expressed on all nucleated cells; class II antigens are expressed on B lymphocytes, activated T lymphocytes, some monocytes/macrophages and dendritic cells. Human endothelium or epithelium does not generally express class II molecules but these can be induced rapidly by injury and inflammatory stimuli. In the context of transplantation the expression of both classes of MHC antigens on resident allograft cells is not constant and is modified by the rejection process (258).

The remarkable polymorphism of MHC antigens, due to the presence of many alleles encoded by at least six loci (located on the short arm of chromosone 6 in humans), makes it extremely unlikely that two unrelated individuals will carry identical antigens (261). The direct recognition of intact MHC molecules, although focused on polymorhic MHC epitopes, is also strongly influenced by the presence of peptides bound in the MHC groove. Because a given MHC molecule can bind many different peptides, including some derived from self-MHC, a high number of possible MHC-peptide combinations can occur that provide a great diversity of MHC binding regions for presentation to the T cell pool. Further it has been speculated that, in addition to the vast number of MHC alloantigen epitopes, the natural high affinity of the T cell receptor-MHC interaction is a key determinant in defining the characteristic intensity of the allogeneic immune response. The histocompatibility immune response is as much as 100-fold stronger than the response to the more common environmental antigens e.g. viruses. It is not surprising, therefore, that organ rejection is the ultimate result.

Allorecognition

It is generally accepted that there are two principal mechanisms of allorecognition; the so-called direct and indirect pathways (262). In the former, T cells recognise intact allo-MHC molecules on the surface of donor cells; peptides derived from endogenous proteins bound in the antigen binding groove of the MHC play an important role in this mode of recognition. In the indirect pathway, self antigen presenting cells (APC) process allo-MHC molecules and present the resultant peptides in the context of their own MHC for T cell recognition. Both mechanisms of

recognition initiate the immune response that characterises the rejection process (Fig 1.11).

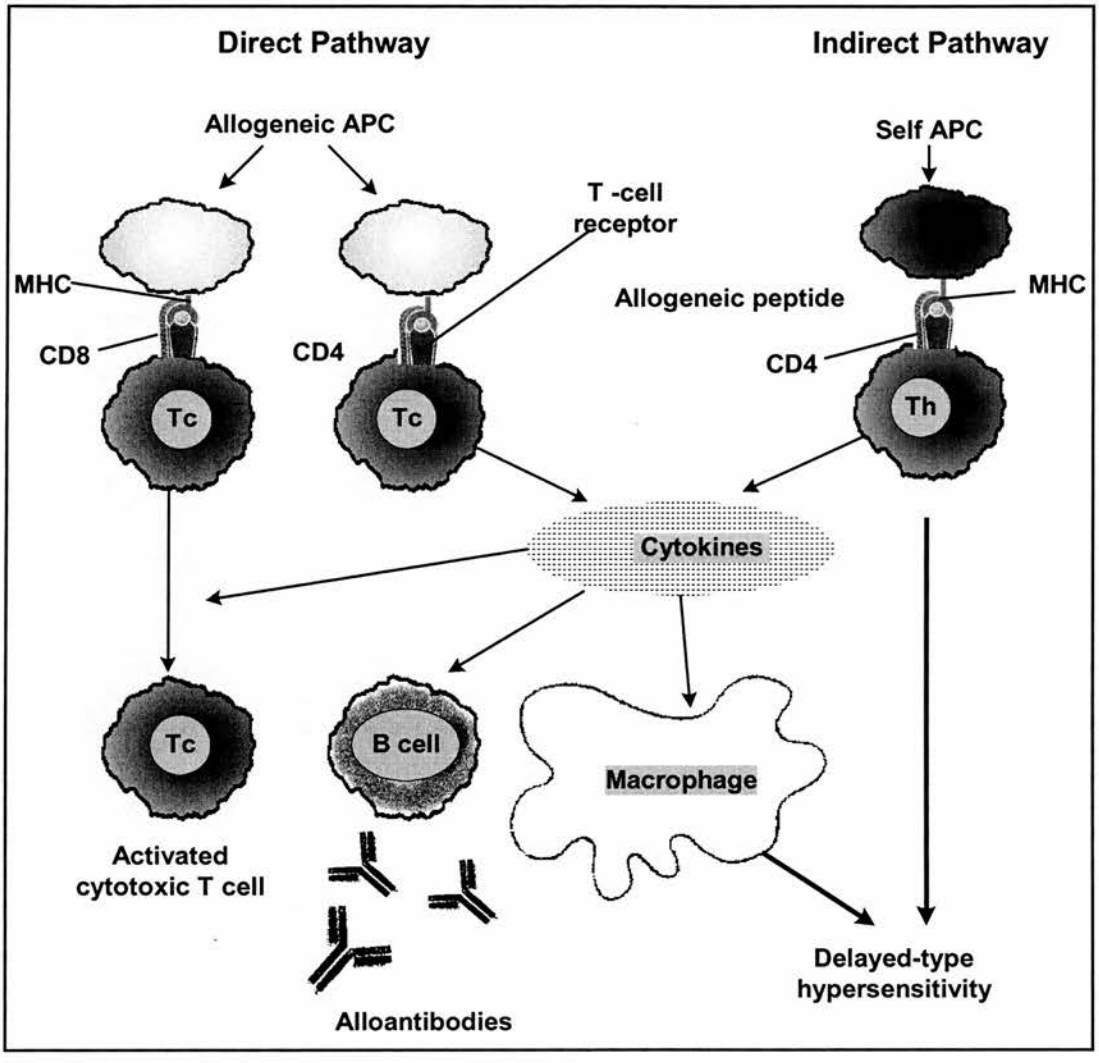


Figure 1.11 Allorecognition: direct and indirect mechanisms
(See text for details. Adapted from reference (263))

Allograft rejection: T helper cells

Two major subsets of T cells, the $CD8^+$ cytotoxic T cells (Tc) and $CD4^+$ T helper (Th) cells recognise processed antigen on MHC class I and class II, respectively (Fig 1.11). The activation of a T cell requires two principal signals. One signal is transduced by the antigen specific T cell receptor when it recognises the antigen in

the context of MHC molecules on the APC and this defines the specificity of the T cell response. The second signal is mediated, via a separate pathway, by a co-stimulatory molecule that is independent of antigen. The best characterised costimulatory molecule is CD28 which is expressed on all T helper and some cytotoxic T cells and binds a family of counter receptors termed B7 expressed by the APC (263). Both pathways interact at a transcriptional level to regulate gene expression and, hence, T cell activation and function. One of the main effects of these signals is to induce the Th cell to produce interleukin (IL)-2, a cytokine that activates the Th cell, enabling it to proliferate and interact with B cells and cytotoxic T cells (Fig 1.12).

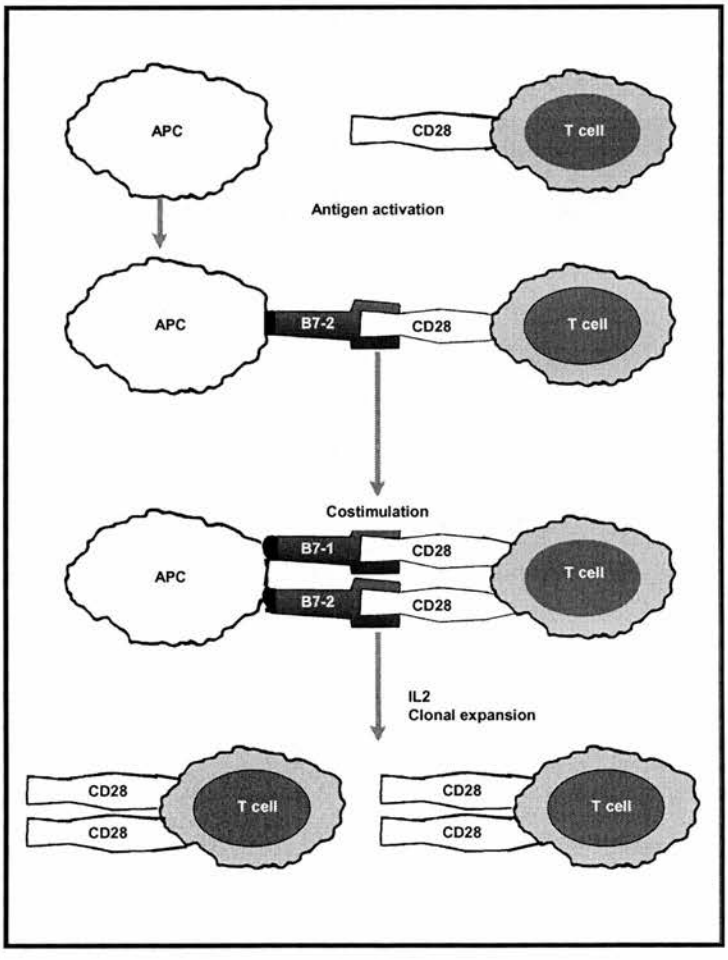


Figure 1.12 T cell co-stimulation: CD28-B7 interactions (from Reference (263).)

CD40 is a member of the TNF receptor family and is expressed on most immune cells and endothelial cells (264). It binds to CD40L, a type II membrane-protein member of the TNF family, present on endothelial cells, activated B cells, APC and platelets. In the context of cardiac rejection, CD40/CD40L interactions can induce expression of costimulatory molecules (B7) on APC and can activate endothelial cells to express adhesion molecules and cytokines that promote the inflammatory response (188, 265).

In addition to CD28, Th cells express a second higher affinity ligand for B7, CTLA4. In contrast to CD28 this delivers a negative signal to the T cell leading to cell cycle arrest (263). Mice deficient in CTLA4 die of a generalised lymphoproliferative disorder within two or three weeks of birth, highlighting the importance of the CTLA4 "off" signal. Blockade of the CD28-B7 interaction by anti-CTLA4 prevents T cell activation and prolongs allograft survival. (Fig 1.13) (266, 267).

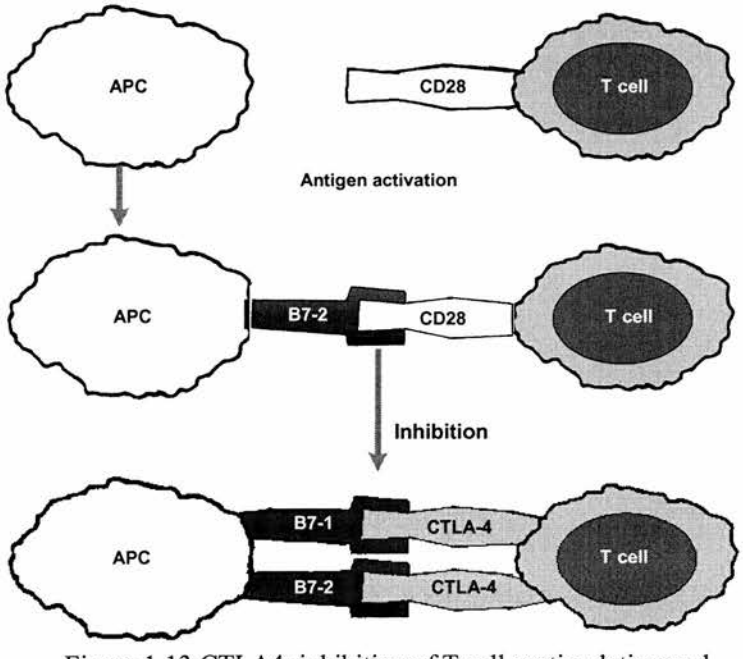


Figure 1.13 CTLA4: inhibition of T cell costimulation and clonal expansion

Allograft rejection: endothelial cells

Endothelial cells of vascularised allografts are the first obligate cells that interact with host lymphocytes. Graft endothelium can influence rejection in two important ways. Firstly, as described above, endothelial cells may participate directly in the activation of alloreactive T lymphocytes via the presentation of alloantigen and effective costimulation of activation. Secondly, the endothelium is critical for the recruitment of T cells into allograft tissue. The movement of immune cells from the blood vessel lumen into allograft tissue is likely to occur via the multistep paradigm of leukocyte-endothelial interactions described previously in section 1.2.1. Endothelial E-selectin expression tends to occur early following transplantation and precedes rejection episodes, whereas ICAM-1/VCAM-1 on endothelial cells tend to be more temporally associated with T cell infiltrates and the rejection process itself (264).

Allograft rejection: chemokines

A large body of evidence suggests that chemokines (discussed in section 1.2.3) participate in leukocyte trafficking into allografts, with documented expression following ischaemia/reperfusion injury, during acute or chronic rejection episodes and in association with viral infections (143). The infiltrating leukocyte population is defined both by the nature of the chemokines secreted by, as well the specific chemokine receptors expressed on the cytokine-activated allograft cells and the infiltrating cells themselves. The elaboration of cytokines and chemokines by T_H, therefore, sustains and amplifies the immune response and is a critical determinant of graft rejection.

Allograft rejection: cytokines

While signalling through the T cell receptor and co-stimulatory molecule is sufficient to activate the T cell to produce and secrete IL-2, these signals alone are not sufficient to promote T cell proliferation. Cytokines affect the proliferation, differentiation and the functioning of immune cells in numerous physiological processes and are central to the generation of an immune response (261). These mediators are produced by multiple different cell types including T cells, B cells, monocytes/macrophages and APC as well as various non-immune cells such as endothelial cells. Subsets of Th cells, namely Th1 and Th2 cells, which exhibit characteristic cytokine production profiles are central to the immune response (268).

Th1 cells exert broadly pro-inflammatory actions via their secretion of IL-2, TNF-α and IFN-γ (269). As previously mentioned IL-2 is a powerful autocrine T cell growth factor, activating T cells, stimulating their growth and giving rise to clonal expansion. By contrast, TNF-α and IFN-γ on the other hand exert activating effects on monocytes and resident cells. These cytokines induce class I and class II antigen expression, modulate adhesion molecule expression and stimulate the release of further cytokines, chemokines and growth factors from monocytes, T cells and endothelial cells. Interleukin IL-1 production by macrophages activated by TNF-α and IFN-γ further enhances the immune response by activating T cells, promoting chemotaxis of additional macrophages into the allograft and by stimulating growth factor and chemokine production by resident cells. Th2 cytokines, principally IL-4, IL-6, IL-10 and IL-13 exert anti-inflammatory effects via their suppression of Th1 cell activation. It is important to note however that Th2 cytokines can "switch on" humoral immnuity via their activation of B lymphocytes. Thus, in the context of

allograft rejection, cytokines both sustain and amplify the immune response by promoting lymphocyte activation, increasing adhesion molecule expression, and regulating MHC expression (268, 269).

Allograft rejection: cytotoxic T cells

In addition to the activation and proliferation of Th cells, the generation of allograft specific $CD8^+$ cytotoxic T cells which recognise donor antigens in the context of MHC Class I results in further destruction of graft tissue (261). Multiple mechanisms may come into play in cytotoxic lymphocyte mediated target cell destruction. Certain cytotoxic lymphocytes have within their cytoplasm lytic granules containing pore forming material. Following binding to target cell lytic granules fuse with the T cell membrane and release their contents into the lymphocyte-target cell intercellular space. Among the granule constituents are the pore forming protein perforin, lysosomal enzymes and serine esterases termed granzymes. It has been speculated that, in the presence of calcium ions, perforin forms non-selective trans-membrane pores resulting in disruption of electrochemical gradients and subsequent colloid osmotic collapse of the affected cell. Cytotoxic T cell-dependent apoptosis of resident cardiac cells is also thought to occur (270). There is now substantial evidence that cytotoxic $CD8^+$T cells can also produce produce cytokines, such as IL-2 and IFN-γ, at levels sufficient to provide autocrine growth. Thus in some circumstances $CD8^+$ cells may provide their own help and function independently of the Th cell subset (258).

These various processes underlying allograft rejection are shown schematically in the figure below (Fig 1.14)

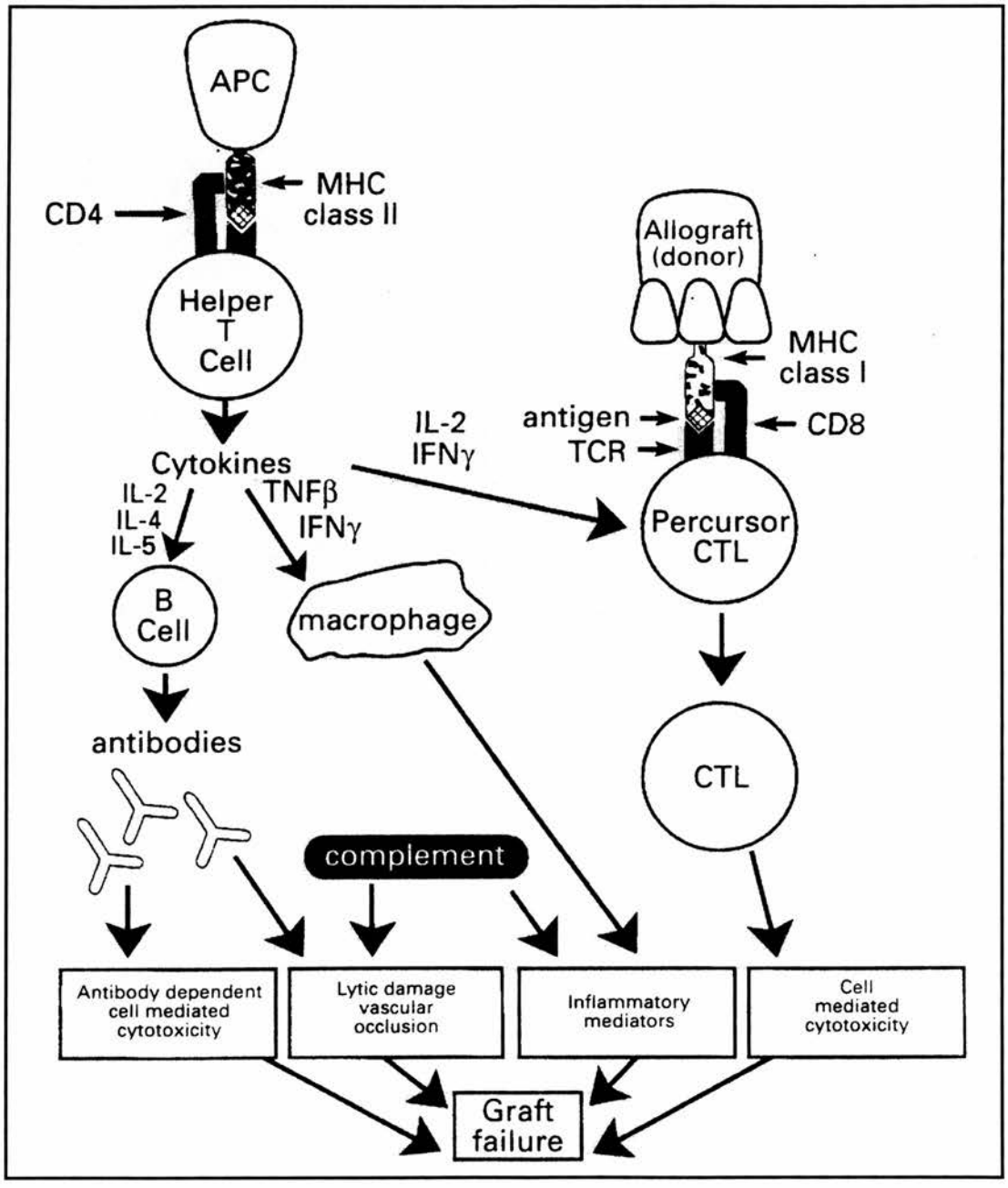


Figure 1.14 Allograft rejection (261).

Schematic illustration of the immunological components of rejection. Activated CD4 T helper cells proliferate and secrete a variety of cytokines that serve as growth and activation factors for CD8 cytotoxic T cells, B cells and macrophages, which cause destruction of the graft by direct lysis of target cells, antibody production and cell-mediated cytotoxicity.

Types of Transplants

Transplanted organs are divided into four categories based on their origin and, hence, genetic relation to the recipient.

1. Autogeneic graft. This is performed within the same individual and is not rejected e.g when skin, blood vessel or bone is moved from one site and grafted to another.

2. Syngeneic graft. A graft is transferred within genetically identical individuals e.g. identical twins or an inbred animal colony and is not rejected.

3. Allogeneic graft. A graft is transferred between individuals belonging to the same species but differing in their genetic repertoire. This is the commonest mode of transplantation in both humans and experimental animals and is frequently associated with rejection.

4. Xenogeneic graft. A graft is transferred from one species to another and is invariably associated with acute rejection.

Clinical syndromes of graft rejection

The rejection of cardiac allografts can be divided into three clinical syndromes on the basis of the pathological process and the kinetics of the rejection response: hyperacute, acute and chronic rejection (reviewed in (258)).

1. Hyperacute rejection.

This occurs within minutes to hours and is caused by pre-existing antibodies to graft antigens, usually AB blood group or HLA class I molecules. Antibodies binding to vascular endothelium of the graft activate complement and cause cell destruction. Activation of endothelial cells also results in alteration in the expression of adhesion molecules which in turn facilitates the movement of phagocytic immune cells into the graft tissue, as well as the activation of platelets and fibrin deposition/thrombus

formation. Current protocols for blood group matching and the screening for prior sensitisation against HLA in recipients have greatly reduced the incidence of hyperacute rejection, but once initiated this process is generally irreversible and results in graft oedema, haemorrhage, vascular thrombosis, ischaemia and, ultimately, allograft rejection. With the continuing shortfall in donor grafts xenotransplantation is once more emerging as a possible alternative, e.g. pig to human transplants, and much work is presently focusing on the development of techniques to avert hyperacute xenograft rejection.

2. Acute rejection

Episodes of acute rejection in humans commence 3 to 7 days after transplantation and occur with decreasing frequency after three months. Acute rejection can, however, occur months to years after transplantation and is frequently associated with the withdrawal of immunosuppressive regimes. The effector mechanisms of graft destruction in acute rejection are multiple and involve cell- mediated cytotoxicity by $CD8^+$cytotoxic T cells, activation and proliferation of $CD4^+$ Th cells, cytokine production, and macrophage activation. The 3-7 days required for the initiation of this process is the time required for presentation of donor antigen by antigen presenting cells in the lymph nodes and spleen, the activation and proliferation of graft specific host T cells and the differentiation, migration and infiltration of effector cells back into the graft. Ischaemia-reperfusion injury associated with the harvesting of a donor graft and its subsequent revascularisation, is also a determinant in graft outcome (271). The response to injury results in the upregulation of MHC molecules, adhesion molecules and increase in expression of

cytokine and growth factor genes. This in turn results in more immune recognition and promotion of the allograft directed immune response.

3. Chronic rejection

A steady rate of attrition of allografts occurs months to years after transplantation and is attributed to chronic rejection (258, 261). The exact immune mechanisms remain poorly understood and pathogenesis appears to be multifactorial. Mild to moderate lymphocytic infiltrates lend support to a cell-mediated process while in some cases graft specific antibodies have been detected. Non-specific factors which are not antigen driven e.g. ischaemia-reperfusion injury are also important. Activated graft infiltrating T cells/macrophages, as well as activated resident endothelial cells secrete growth factors such as platelet derived growth factor (PDGF) and fibroblast growth factor (FGF) which stimulate smooth muscle cell proliferation. The resultant sustained activation of the inflammatory response, despite immunosuppressive therapy, results in interstitial fibrosis and intimal proliferation in arteries and arterioles i.e. graft atherosclerosis (272). Cardiac transplants are particularly sensitive to the vascular changes of chronic rejection because of myocardial dependence on coronary blood flow with ensuing graft dysfunction and cardiac failure.

In practice these syndromes of rejection are frequently overlapping and are often not totally distinct. All forms of rejection involve a combination of antigen-specific cell-mediated mechanisms, humoral mechanisms, cytokine elaboration and non-immune factors such as ischaemia, infection, age and comorbidities in both donor and recipient. It is generally accepted, however, that the degree of MHC mismatch correlates positively with the degree of acute rejection and that the number of acute

rejection episodes is a predictive factor for the subsequent development of chronic rejection.

1.3.6 Cross-over phenotype

It is apparent that, among these various phenotypes, shared mechanisms of vascular pathogenesis exist. The inflammatory response, while primarily intended to neutralise the initiating and "injurious" stimulus and promote healing and repair, can itself result in extensive tissue damage and organ dysfunction when inappropriate and sustained activation occurs. Inflammation is a complex, highly orchestrated process involving many cell types and molecules, some of which initiate, amplify or sustain the process, some of which attenuate it, and some of which promote its resolution. Although the initiating stimuli may differ, many of these diverse phenotypes share certain features at a cellular level: vascular cell activation and subsequent cell adhesion molecule upregulation, leukocyte infiltration of the vessel/tissue and the elaboration of cytokines and chemokines. It is not surprising therefore that some of these conditions also share certain features at a phenotypic level. The association, for example, between atherosclerosis and thrombosis has led to the term atherothrombosis which emphasises the importance of atherosclerotic plaque in the formation of thrombi and vice versa (273). Ischaemic acute tubular necrosis is mediated by a combination of vasoconstriction and inflammation occuring within the kidney, while hyperacute rejection on the other hand represents a cross-over between the alloreactive and thrombotic phenotypes. Many additional examples of such cross-over phenotype exist but the following discussion will focus specifically on the above.

The nature of the chronic inflammatory response underlying atherogenesis is discussed in detail in section 1.3.3. Individual atherosclerotic lesions often fall into two categories: stable and vulnerable. Both types may coexist within a given arterial

tree. Stable plaques, which are often more stenotic, may cause ischaemia and, hence, produce warning symptoms before thrombotic complications occur. Disruption of vulnerable plaques, however, exposes thrombogenic surfaces within the plaque to circulating blood and may result in thrombotic occlusion of the affected vessel, giving rise to an acute coronary syndrome or myocardial infarction. In cigarette smokers these effects are enhanced by an impairment of acute endothelial tPA release, a crucial event if endogenous fibrinolysis within the vessel is to be effective (274). Plaque stability is determined by the composition (and hence strength) of the fibrous cap. Several inflammatory mediators (section 1.3.3), by modulating vascular cell production of matrix metalloproteinases (which degrade all major plaque constituents), can contribute to weakening of the fibrous cap and its subsequent rupture (186). In addition, mural thrombi can be incorporated into the plaque, enhancing the evolution of atherosclerotic lesions by the release of coagulation factors, such as thrombin, which can promote vascular smooth muscle proliferation and cytokine release (273). For these reasons, most therapeutic regimes for patients with ischaemic heart disease incorporate an anti-platelet agent in addition to standard anti-anginal therapy.

Elevated plasma homocysteine levels are associated with premature atherosclerosis in the coronary, cerebral and peripheral vasculature, as well as recurrent venous and arterial thrombosis (234). Evidence suggests that the atherogenic propensity associated with hyperhomocysteinemia is a consequence of endothelial dysfunction and injury, followed by platelet activation and thrombus formation. Although the exact mechanisms underlying homocysteine-mediated endothelial dysfunction are unknown, there is growing evidence that homocysteine exerts its effects by

promoting oxidative damage. Homocysteine is rapidly oxidised with the subsequent generation of potent reactive oxygen species and this process has been shown to support the oxidation of LDL. In addition, homocysteine alters the normal anti-thrombotic phenotype of the endothelium by enhancing the activities of factor XII and factor V and depressing the activation of protein C. Homocysteine also inhibits the expression of thrombomodulin, induces the expression of tissue factor and suppresses the expression of heparin sulphate by the endothelium. All of these effects facilitate the formation of thrombin and create a prothrombotic environment, thereby, and at least partially, explaining the propensity to both venous and arterial thrombosis characteristic of this disease.

A wide spectrum of clinical conditions result in a generalised or localised reduction in renal blood flow. These include intravascular volume depletion such as occurs in haemorrhage/GI fluid losses, decreased effective intravascular volume e.g. congestive cardiac failure/cirrhosis, large and small vessel renal vascular disease and a variety of medications and toxins. In the ischaemic kidney, the local production of inflammatory mediators such as cytokines, complement-activation products, platelet activating factor and leukotrienes is associated with increased expression of endothelial adhesion molecules such as ICAM-1 and selectins (251). This upregulation facilitates the extravasation of neutrophils into ischaemic tissue. Interestingly, in animal models of renal, myocardial or intestinal ischaemia the depletion of neutrophils, blockade of neutrophil adhesion to the endothelium and inhibition of the complement system all reduce tissue injury (275-277). In addition intrarenal vasoconstriction may result from a variety of vasoactive agents acting on the blood vessels of the kidney. Such insults can be systemic e.g. cyclosporin,

tacrolimus and radioactive contrast agents - all of which result in vasoconstriction of small renal vessels, or local e.g vasoactive eicosanoids such as leukotrienes. The resulting ischaemia can directly alter endothelial cell function with subsequent imbalance in the endothelial production of, and response to, vasoactive mediators. Such vasoconstriction, when combined with enhanced local inflammatory responses, may aggravate injury in the post ischaemic kidney.

Ischaemic injury to an allograft can lead to delayed graft function which has been associated with acute rejection and decreased graft survival (258). Hyperacute rejection occurs within minutes to hours and is caused by pre-existing antibodies to graft antigens usually AB blood group or HLA Class I molecules (258). Antibody binding to the vascular endothelium of the graft can result in complement activation and cell destruction. Endothelial cell activation also results in increased adhesion molecules expression which, as has been discussed, facilitates the movement of immune cells into the graft tissue. In addition, the activation of platelets and fibrin deposition/thrombus formation results in a thrombotic microangiopathy. This cross-over between the alloreactive and thrombotic phenotypes results in graft oedema, haemorrhage, vascular thrombosis, ischaemia and, ultimately, allograft rejection.

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Chapter 2

Mechanical Strain: A trigger for antigen – independent vascular cell activation in systemic and glomerular hypertension

Chapter 2

Mechanical Strain: A trigger for antigen – independent vascular cell activation in systemic and glomerular hypertension

2.1. Pathophysiology of systemic hypertension

2.1.1. Overview

An elevated arterial blood pressure is common, generally asymptomatic, and often leads to lethal complications if left untreated (1). Patients with hypertension are at increased risk for a variety of cardiovascular sequelae including angina pectoris, myocardial infarction, stroke, heart failure and sudden death, in addition to renal and retinal complications (2). Clinical studies have demonstrated that treatment of this disorder reduces the incidence of such complications. In 90% of cases the aetiology of hypertension is still largely unknown and a diagnosis of so-called essential hypertension is made after exclusion of the known causes (most commonly renal and adrenocortical disorders) (3). It is probable that essential hypertension itself does not arise from a single pathology, but rather is the clinical presentation of a number of different disease processes which can each result in elevated blood pressure. The end result, however, despite pathophysiologically divergent aetiologies, is a relatively uniform effect on the arterial wall in terms of both vascular hypertrophy and the development and progression of atherosclerosis.

In long-standing essential hypertension, the fundamental abnormality is an increase in peripheral vascular resistance due to a decrease in lumen diameter in small arteries, arterioles and pre-capillary sphincters (resistance vessels). This occurs even when vessels are maximally dilated, indicating an underlying alteration in vessel

structure rather than functional "overactivity." A complex interplay between genetic and environmental factors which control cell growth and the neurohormonal milieu within the vasculature, results not only in medial smooth muscle hypertrophy/hyperplasia, but endothelial dysfunction with reduced endothelium-dependent relaxation (4). The subsequent decrease in vessel lumen diameter produced by these changes leads to increases in peripheral vascular resistance and, ultimately, hypertension. With the development of hypertension, ongoing changes in the production of vasoactive mediators and alterations in the various haemodynamic forces within the vascular lumen, promotes further structural changes in a self-perpetuating process. (Fig 2.1)

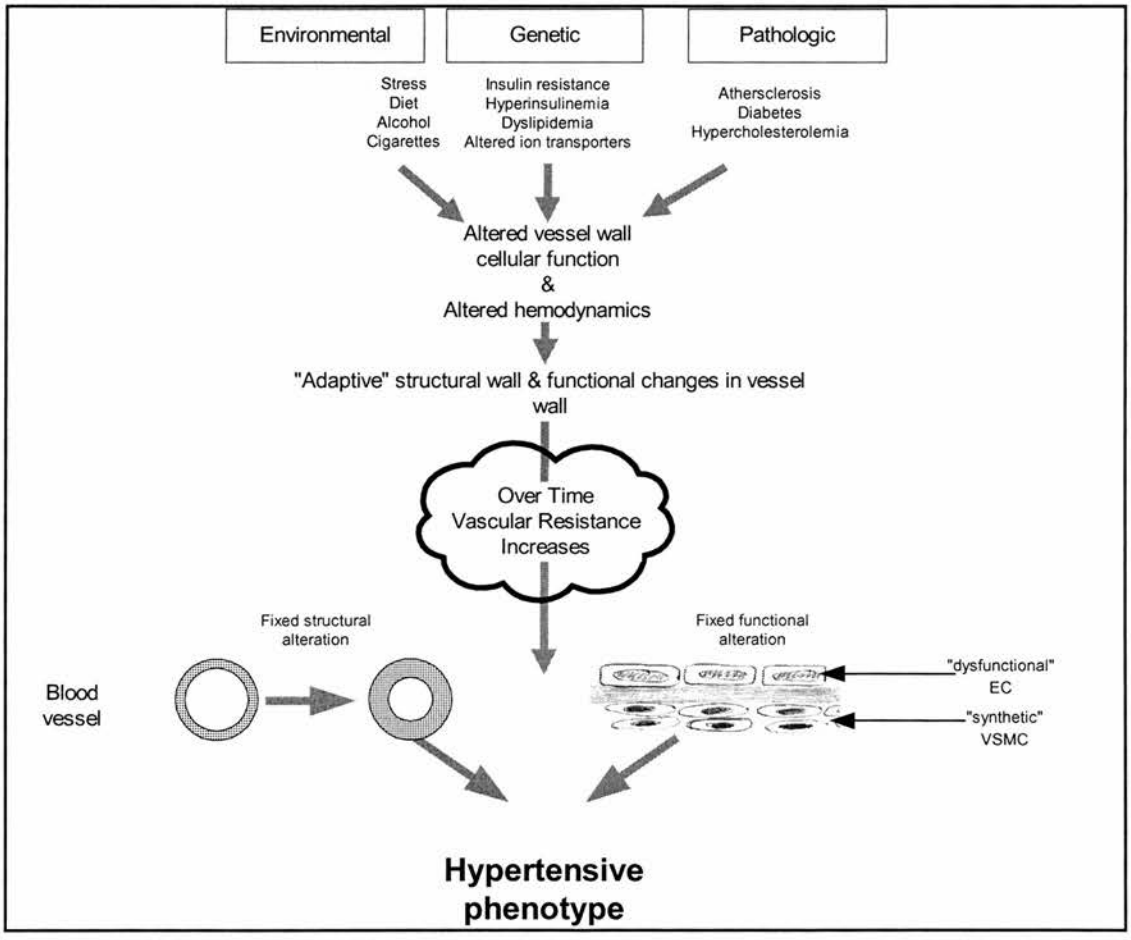


Figure 2.1 Hypertensive vessel wall: structural and functional characteristics

(adapted from(4))

The endothelium in hypertension

The concept that endothelial function is disturbed in hypertension and perpetuates high blood pressure, remodelling and atherogenesis, relies on a number of observations. The best characterised alteration is diminished endothelium-dependent relaxation in response to acetylcholine. This has been demonstrated in both mesenteric vessels of hypertensive rats (5) and in the forearm circulation of hypertensive patients (6). This dysfunction is related to a decrease in the effective concentration of endothelial derived relaxing factors (EDRF), the most important of which is nitric oxide (NO). NO has been discussed in detail in section 1.1.1 and it has been established that in the hypertensive state there is decreased basal production of NO (7). In addition, however, administration of superoxide dismutase (an enzyme that destroys the superoxide radical) decreases blood pressure in spontaneously hypertensive rat (SHR), leading to speculation that the presence of increased free radicals in hypertension may result in increased destruction of NO. Finally, NO exerts its vasodilating effects by increasing cGMP which inhibits myosin phosphorylation and subsequent smooth muscle contraction. It appears that this series of events may be altered in smooth muscle cells exposed to chronic hypertension, as shown by the decreased relaxation in the aorta and carotid of the SHR on administration of a variety of compounds that donate NO, e.g. nitroprusside, and thereby stimulate cGMP (8).

In addition to decreased nitric oxide production, the endothelium may release increased amounts of constricting factors such as the potent vasoconstrictor endothelin-1 (ET-1). ET-1, secreted mainly by the endothelium but also by vascular smooth muscle cells, acts in a paracrine or autocrine fashion on blood vessels by

binding to endothelin receptors A and B (ET_A and ET_B) on smooth muscle to mediate contraction (9). This effect is modulated by endothelial ET_B receptors where ligand binding results in production of the vasodilators NO and prostacyclin. Production of ET-1 is enhanced in several experimental rat models of hypertension (10), and while plasma ET-1 levels are generally not elevated in essential hypertension, there may be an increase in its local concentrations. Endothelin-dependent vascular tone has been demonstrated in studies of forearm blood flow and enhanced expression of ET-1 mRNA has been shown in the endothelium of small arteries of patients with moderate to severe hypertension (11).

2.1.2. Vascular smooth muscle cells (VSMC) in hypertension

Increases in vessel wall thickness in hypertension result from increases in smooth muscle cell size (cell hypertrophy), increases in smooth muscle cell number (cell hyperplasia) and increases in matrix deposition (4). These changes are initially adaptive in that they act to return the increased wall stresses occuring in hypertension to normal. Furthermore, the increased macrovascular pressures in hypertension, if transmitted unabated to arterioles and capillaries, could potentially damage the endothelium of the microcirculation. This is particularly critical in the cerebral circulation where increased vascular permeability would rapidly lead to cerebral oedema. VSMC (section 1.1.2) are clearly a heterogeneous group. Contractile VSMC are spindle-shaped, located in the media, contract in response to agonists and express high levels of the contractile proteins α-actin and smooth muscle cell specific myosin. Synthetic phenotype VSMC are round, may be present in the intima, do not contract and do not express α-actin and myosin. These cells are characterised by large amounts of rough endoplasmic reticulum, secretion of large amounts of tissue

matrix proteins and a proliferative phenotype *in vitro*. Contractile smooth muscle cells, by contrast, do not initially proliferate in culture, but can undergo a process of phenotypic modulation in which they take on the appearance of synthetic cells and begin to proliferate in response to mitogens.

Various growth factors have been implicated in VSMC responses to elevated pressures and the following discussion will focus on the most important of these.

It has now been established that the entire renin-angiotensin system is present within the wall of normal vessels and its activity is dynamically regulated, resulting ultimately in the production of angiotensin II (AT II) (12). AT II functions (predominantly through the AT II type 1(AT 1) receptor) as an acute vasoconstrictor, regulating systemic blood pressure and vascular tone. Furthermore AT II is also involved in cardiac and vascular growth processes (13). It induces smooth muscle hypertrophy *in vitro* through increased expression of PDGF-A, TGF-β and the proto-oncogenes *c-myc, c-fos* and *c-jun* (14). AT II-induced VSMC growth appears to be mainly due to PDGF production, as was evidenced by the attenuation of AT II dependent effects by antisense oligonucleotides to PDGF-A mRNA (15). In this study, TGF-β1 appeared to negatively modulate the mitogenic effect of PDGF since, in the presence of neutralising antibody to TGF-β1, PDGF treatment of rat VSMC resulted in enhanced AT II-induced DNA synthesis and proliferation of these cells. In the light of these findings, it has been speculated that AT II is a bifunctional growth factor, capable of activating (through PDGF) or modulating (through TGF-β) cellular growth.

The concept that the renin-angiotensin system may be of crucial importance in both hypertension and atherogenesis has recently gained support from studies of candidate genes of hypertension (14). Genetic analysis of a restriction fragment length polymorphism of genomic DNA has recently established a linkage between polymorphisms of the angiotensin-converting enzyme (ACE) gene and salt-induced hypertension in stroke-prone SHRs. Although conflicting reports on such an association exist in human essential hypertension, a deletion polymorphism of this gene results in higher plasma ACE levels in affected subjects and appears to confer an increased risk of related pathologies including atheroscelerosis, myocardial infarction and sudden death. Two allelic variants of the angiotensinogen gene have been linked to hypertension in a sib-pair linkage analysis of two ethnically different populations, and in one of these variants there was increased angiotensinogen levels. Interest recently has focused on polymorphisms of the AT I receptor, and indeed an A-C tranversion at nucleotide position 1166 located in the 3'untranslated region of the AT1-receptor gene($A^{1166}C$) is associated with human essential hypertension (16). No clear human phenotype has yet been identified with this genotype.

Other classic vasoconstrictors such as catecholamines have also been implicated in hypertension. In patients with pheochromocytoma a marked increase in plasma catecholamine levels are associated with both sustained and episodic hypertension. *In vitro* noradrenaline stimulates VSMC hypertrophy and hyperplasia (17), and in carotid injury models α1-adrenergic stimulation caused PDGF A chain expression (18). As yet however no convincing evidence exists which demonstrates a role for abnormal catecholamine secretion or metabolism in human essential hypertension.

A number of peptide growth factors are synthesised locally in the blood vessel and are involved in VSMC growth. As has been discussed, PDGF is a potent smooth muscle cell mitogen. PDGF is a heterodimeric protein composed of two distinct polypeptide chains, PDGF-A and PDGF-B. Elevated PDGF-A mRNA levels have been demonstrated in both small and large blood vessels in various rat models of hypertension (14). Furthermore, the recent finding of increased plasma levels of PDGF-AB and PDGF-BB in patients with never-treated mild hypertension suggests a role for PDGF in the vascular changes associated with this process (19).

Insulin-like growth factor 1 (IGF-1) promotes hypertrophy and matrix deposition by VSMC *in vitro*, and also enhances the mitogenic effects of PDGF. IGF-1 mRNA expression is increased in the rat aorta above the coarctation site in infrarenal aortic coarctation, although the precise effect of this increase remains to be determined (20). IGF-1 mRNA expression is also induced in VSMC in vitro in response to AT II, suggesting that IGF-1 may participate in the AT II growth pathway. This may be of particular relevance to those patients with a strong genetic predisposition to a combination of essential hypertension, insulin resistance and hyperinsulinaemia. Whether insulin acts as a growth factor via its own receptors, or via the IGF-1 receptors for which it has a relatively weak affinity, remains to be determined.

ET-1, in addition to causing vasoconstriction, can also function as a mitogen for VSMC in vitro. However when administered in serum-free conditions ET-1 displays only weak mitogenic activity, and it has been speculated that its importance is as a co-mitogen with other growth factors present in the vessel wall (21).

2.1.3. Extra-cellular matrix – changes in hypertension

The extra-cellular matrix (ECM) is a complex protein-carbohydrate network consisting of collagens, elastins, proteoglycans and glycopeptides which is a critical regulator of blood vessel function and its physical characteristics The composition of the matrix influences how vascular cells percieve the forces applied to the vessel wall. This role takes several forms: firstly the three dimensional structure of the matrix and the resulting "stiffness" influences the extent to which cells within the matrix may be physically deformed. Cells within a highly collagenous vessel for example may be less susceptible to deformation than those in a more elastic vessel. Secondly, the matrix may influence the alignment of cells within it, biasing the extent and direction of the force to which cells in the vessel wall are exposed. Finally all vascular cells are able to generate tension through their cytoskeleton, and this ability to contract and the tension generated depends on the nature of the matrix surrounding the cells. Forces generated by a cell as it contracts can influence its cellular activity and response to growth factors. Increases in collagen, elastin and fibronectin levels have been demonstrated in the aorta of hypertensive rats (4).

Production of these molecules is regulated by a variety of enzymes and growth factors produced by VSMC and endothelial cells that alter both the structure of the ECM and its ordered dissolution and reassembly. Collagenase activity, for example, is tightly regulated in VSMC. Expression of VSMC collagenase *in vitro* is stimulated by growth factors via a phorbal ester response element, while heparin limits its induction (22). Proteases such as urokinase and tissue plasminogen activator are induced during mitogenesis of VSMC *in vivo*, and their expression, at least *in vitro,* is also blocked by heparin (23). The composition of the ECM may also influence

smooth muscle cell phenotype. Culture of VSMC on a fibronectin matrix, for example, promotes the adoption of a synthetic proliferative phenotype when cells are placed in culture, whereas laminin maintains smooth muscle cells in a contractile form for a prolonged period (24). The binding of fibrogenic growth factors such as TGF-β and FGF to matrix components also regulates their activity. Binding of TGF-β to the matrix protein decorin results in the inactivation of this cytokine, while the binding of FGF to ECM heparin-sulphate proteoglycans is thought to provide both a reservoir of FGF and, in addition, protect this growth factor from proteolytic degradation (25).

The ECM therefore is a critical regulator of vessel growth and structure by virtue of its own dynamic regulation as well as its ability to modulate the activity of both growth-promoting and growth-inhibiting factors. Although, to date, the hypertension-associated changes occuring in the composition of the extra-cellular matrix or its growth-factor binding capacity remain largely undefined, it is obvious that any alterations in these factors could have profound effects on both vessel structure and the phenotypic expression of VSMC, thereby determining the vascular remodelling that is pathognomic of this disease.

2.1.4. The hypertensive phenotype

Several abnormalities of signal transduction and ion transport leading to increased VSMC responsiveness to growth factors and alterations in sensitivity to contractile agonists have been proposed in the development of the hypertensive phenotype. One such abnormality - enhanced Na^+/H^+ exchange - is present consistently in both human essential hypertension and animal models of hypertension (4).

The growth-activated Na^+/H^+ exchanger (NHE-1) is a member of a multigene family which exchanges one H^+ ion for one Na^+ ion when decreases in extracellular pH occur. In addition increases in intracellular Na^+ occur via this exchanger in response to cell shrinkage, restoring cell volume by obligate water entry. It appears that the NHE-1 participates in signal transduction pathways by which vasoactive agents modulate vascular tone and regulate smooth muscle proliferation. Evidence for dysfunction of the exchanger in hypertension is provided by the observation that its activity is increased in cells from hypertensive patients (26). In addition, several studies indicate that both cultured VSMC and intact mesenteric arteries from SHR demonstrate enhanced exchanger activity (27). To date there is no evidence for mutations or altered regulation of this gene in hypertensive populations, and studies in the SHR have demonstrated no changes at mRNA level. It may be then that this enhancement in exchanger activity may be a consequence of post-translational modification Such increased exchange activity may result in an enhanced sensitivity to mitogens, thereby facilitating VSMC proliferation. Increased vascular tone secondary to this elevation in NHE-1 activity could occur by two potential mechanisms. First, increased Na+ entry into the cell could activate Na^+/Ca^{2+} exchange, leading to increased intracellular calcium. Second, increased intracellular pH would enhance the Ca^{2+} sensitivity of the contractile apparatus leading to an increase in contractility for a given intracellular Ca^{2+} concentration (28).

In addition many alterations in *downstream* effectors have also been demonstrated in SHR-derived cells as compared to cells derived from their normotensive counterparts (4). For example, phospholipase C-mediated phosphoinositide turnover is stimulated

by significantly lower concentrations of thrombin and angiotensin II in the SHR. Increased PKC activity and increased intracellular calcium have also been demonstrated in VSMC from the SHR. The fact that these abnormalities persist in culture has led to speculation that there may be genetic abnormalities in the regulation of these enzymes. Although no such abnormality has been detected to date in human essential hypertension, it may be that enhancement of such intracellular mediators in hypertensive VSMC alters common regulatory events such as ion fluxes and cell cycle genes leading to both increased vessel tone and increased VSMC growth (29).

2.1.5. Hypertension-induced atherosclerosis

The molecular mechanisms underlying atherosclerosis are discussed in detail in section 1.3.1. Hypertension is an established risk factor for atherosclerosis, but the underlying molecular mechanisms have not yet been clearly elucidated. A great deal of experimental, epidemiological and clinical evidence suggests an important role for the renin-angiotensin system in atherogenesis. Hypertensive patients with elevated AT II levels show a five-fold increase in their incidence of myocardial infarction (30). ACE inhibitors reduce atherosclerotic formation in several experimental animal models, such as Watanabe heritable hyperlipidaemia (WHHL) rabbits, cholesterol-fed mice and monkeys (31). In addition, treatment of patients with left ventricular dysfunction with ACE inhibitors, reduces the incidence of recurrent myocardial infarction and sudden death, implying an anti-ischaemic role for these drugs (32). In addition to its vasoactive role, AT II directly induces oxidative stress in the vasculature by generating superoxide anions through the activation of NADH/NADPH oxidase. This has been demonstrated in cultured rat aortic smooth

muscle cells and in aortas of rats made hypertensive by infusion of AT II (33, 34). Moreover, AT II may also contribute to the inflammatory axis of atherogenesis. Treatment of rat vascular smooth muscle cells with AT II resulted in a dose-dependant increase in MCP-1 gene expression, and, in keeping with this observation, MCP-1 mRNA expression was increased almost four-fold in the aortas of rats rendered hypertensive by either AT II or norepinephrine infusion (33, 34). This was accompanied by recruitment of monocytes/macrophages into the media and adventitia of the hypertensive aorta. In a double transgenic rat (dTGR) model of high human renin hypertension in which rats are transgenic for the both the human angiotensinogen and renin genes, rats develop moderately severe hypertension and die of end-organ cardiac and renal failure by week 7 (35). The hearts of the animals are necrotic and fibrotic, whereas vasculopathy in the kidneys resembles that of the haemolytic uraemic syndrome. There is an early increase in cell adhesion molecules (ICAM-1, VCAM-1) in the interstitium, intima and adventitia of small cardiac and renal vessels while leukocyte infiltration into the vessel wall accompanies PAI-1, VEGF and MCP-1 vascular expression. Expression of TGF-β and extracellular matrix proteins then follows. In this model treatment with ACE inhibitors and AT1 receptor blockers lower blood pressure and, when combined, prevent vasculopathy (36). The up-regulation of NF-kB in the kidney of this model has led the authors to speculate that AT II, via activation of this transcription factor, may initiate cytokine and chemokine transcription, leading to the consequent inflammatory cascade. (Fig 2.2[over])

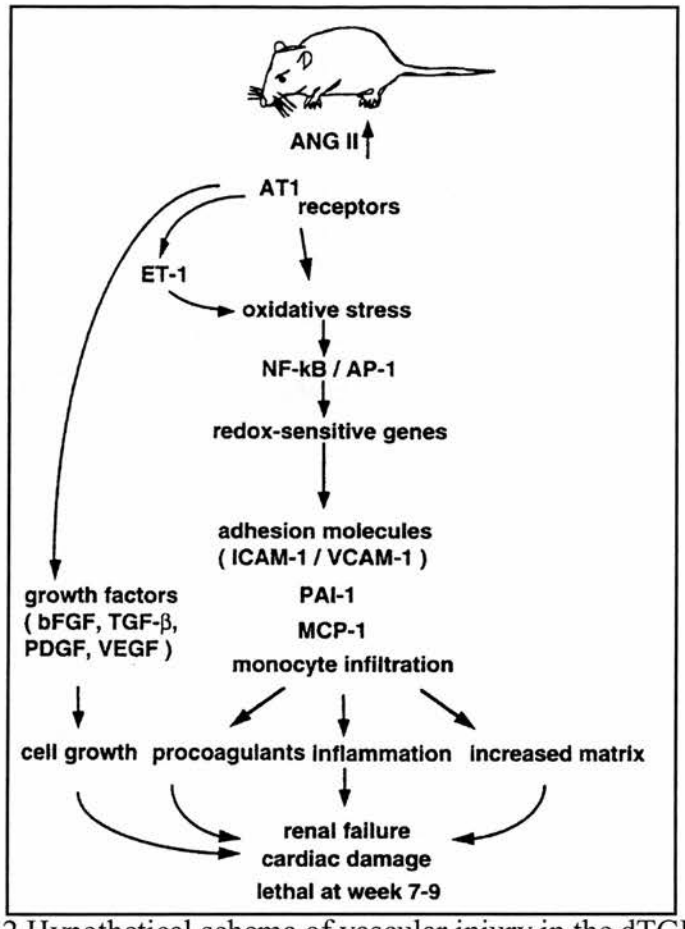


Figure 2.2 Hypothetical schema of vascular injury in the dTGR model.
(adapted from reference (35))

Recent studies also suggest an interaction between hyperlipidaemia, activation of the renin-angiotensin system and atherosclerotic disease (31). LDL upregulates expression of the AT II receptor (AT_1) in cultured VSMC and AT_1 protein expression is increased two-fold in hypercholesterolaemic rabbits. AT II, in turn, facilitates oxidation of LDL and its uptake by scavenger receptors on monocytes/macrophages. The endothelial receptor for ox-LDL, LOX-1, is upregulated in the SHR. It has also been shown that human coronary artery endothelial cells (HCAEC) express the AT1 receptor, and that AT II increases the uptake of ox-LDL by HCAEC *in vitro* in a concentration-dependent manner (37). This increased uptake is due to the upregulation of the LOX-1 receptor on HCAEC. As previously described in section 1.3.1, ox-LDL uptake via LOX-1 on vascular

endothelium may cause endothelial activation/dysfunction, initiating the inflammatory process that is characteristic of atherosclerosis. The molecular mechanisms by which AT II upregulates LOX-1 expression and indeed how ox-LDL injures endothelial cells are, as yet, unknown. Although limited to *in vitro* findings these studies suggest that cross talk between ox-LDL and AT II may be relevant in atherosclerosis and may provide a long-sought molecular link between hypertension, hyperlipidaemia and atherogenesis.

2.1.6. Mechanical deformation of the arterial wall in hypertension: a mechanism for vascular pathology

It has become increasingly apparent that mechanical deformation of the arterial wall is an important proximal signal in the development of hypertensive vasculopathy. The increased haemodynamic forces occuring in hypertension induce expression of a variety of growth-stimulating and pro-inflammatory gene products which mediate both the altered vessel growth characteristic of hypertension and its pro-atherogenic effects (38).

Early *ex-vivo* work demonstrated that increases in luminal pressure of arteries resulted in increased vessel radius and subsequently wall tension. At higher arterial pressures there is a greater incremental increase in wall tension for a given increase in pressure (38). This acute effect can be balanced by the development of vascular hypertrophy, with a resulting increase in wall thickness and a decrease in wall stress. In an attempt to dissociate mechanical and humoral factors workers employed an *in vivo* aortic coarctation model in which the blood pressure below the coarctation remains relatively normal, while above the coarctation it is elevated (39). Although

the entire aorta was exposed to the same humoral milieu, only the aorta above the coarctation demonstrated hypertrophy, implying that mechanical forces contributed to the pathogenic manifestations of hypertension. In another study bifurcating arteries from cholesterol-fed rabbits were cast in acrylic at low pressure to reduce wall stress, thereby facilitating the dissociation of wall strain-dependent effects (40). Arterial segments treated in this way exhibited a complete absence of atherosclerotic lesions. In this model however, atherosclerosis does not normally occur without cholesterol feeding, and therefore, while the authors concluded that wall stress is a necessary component of atherogenesis, it is unlikely that this stimulus alone is sufficient to initiate this pathogenic process.

Available data support the concept that pressure-induced wall stress is a potentially important factor in the development of hypertrophy and atherosclerosis. The pulsatile component of the arterial pressure, and the cyclic alterations in wall stress in response to these pulsatile changes, may also be critical in the development of hypertensive vasculopathy. In animal studies using SHR the media/lumen ratio in resistance vessels (a measure of vascular wall hypertrophy) correlated more closely to pulse pressure than any other parameter studied, including systolic, diastolic or mean arterial pressure (38). In support of this finding, a recent study identified pulse pressure (as opposed to mean, peak or diastolic pressure) as the stongest predictor of all-cause and cardiovascular mortality in 19,000 French males undergoing routine physical examinations (41).

2.2. Biomechanical forces: triggers for endothelial cell activation

Endothelial cells are constantly exposed to a spectrum of haemodynamic forces generated by pulsatile blood flow and, by virtue of their strategic position, are ideally placed to serve both as a sensor of these various signals and as effectors of the biological responses to them (42). *In vivo* vascular walls are exposed to three main haemodynamic forces: (1) shear stress, a tangential frictional force due to blood flow; (2) transmural hydrostatic pressure, a perpendicular cyclic force due to blood pressure; and (3) mechanical stretch, a cyclic tensile stress caused by blood pressure. The recognition therefore that the native condition of the endothelial cells is one of continuous mechanical deformation drove the development of *in vitro* models to investigate these forces and their effects on endothelial function. On the basis of studies in these models, there is increasing evidence that these biomechanical stimuli can directly influence endothelial structure and function, acutely and chronically, thus constituting a novel paradigm of endothelial activation (42, 43)(Fig 2.3).

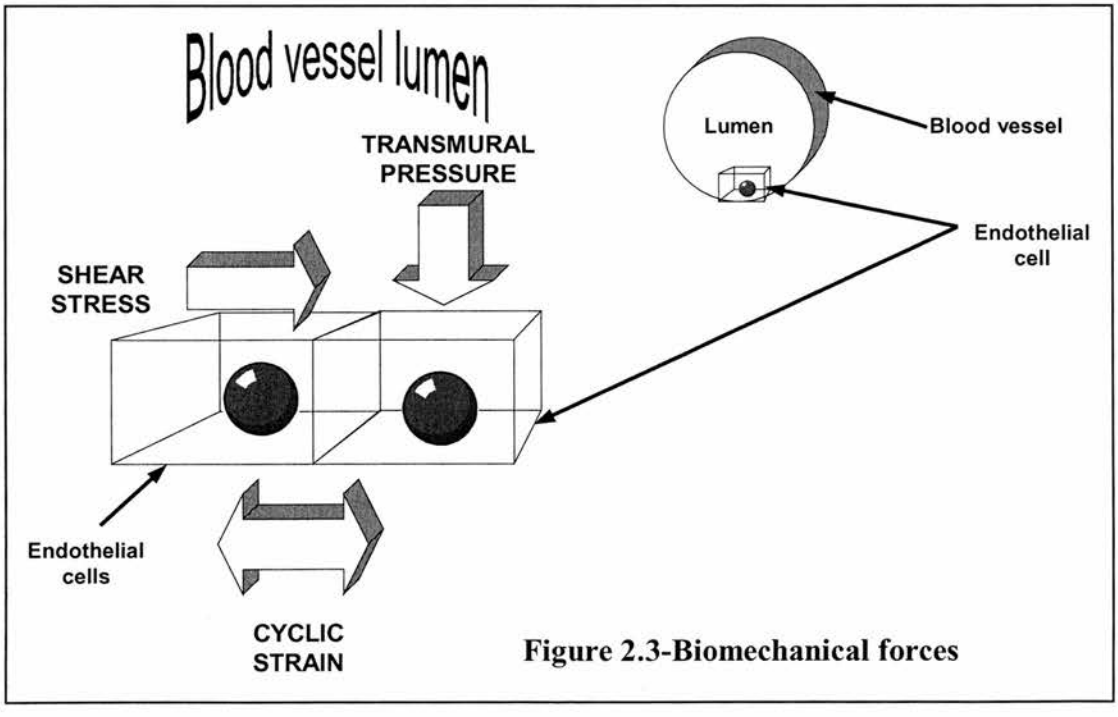


Figure 2.3-Biomechanical forces

2.2.1. Endothelial cells and fluid shear stresses

Shear stress is the frictional force applied along the endothelial cell surface as blood flows through the vessel lumen. This flow can be uniform laminar or turbulent. Investigators have designed flow chambers which can generate various forms of shear stress and facilitate studies into the effects of this force on the endothelium (44). Culturing endothelial cells in dynamic conditions which mimic physiologically relevant degrees of shear results in a spectrum of structural and functional changes (45). These include striking morphological changes in cell shape (polygonal to ellipsoidal), realignment (in the direction of flow) and cell architecture. At a basic cell biology level, many of these changes could be viewed as the *in vitro* readaptation of the cultured endothelial cell to biomechanical stimuli normally present *in vivo*. Real-time visualisation of a cultured endothelial cell monolayer exposed to unidirectional laminar shear stress (LSS) using tandem scanning confocal microscopy reveals a dynamic remodelling of the focal contact sites along its basal aspect (46). Moreover, these focal adhesion complexes, as well as adhesion molecules such as platelet-endothelial cellular adhesion molecule (PECAM)-1 which are localised to lateral cell-cell junctions, have also been shown to demonstrate increased phosphorylation. These internal changes in cytoskeletal architecture and external changes in tomography presumably reflect generalised cellular adaptations to applied mechanical stresses, such that the endothelial cell is in equilibrium with its ambient fluid environment.

In addition to these structural adaptations, biomechanical forces such as shear stress also stimulate endothelial production of a large and diverse array of biological mediators (43, 45). Such responses include the acute release of NO and PGI_2,

activation of transcription factors NF-kB, c-fos, c-jun and SP-1, and the transcriptional activation of genes including ICAM-1, MCP-1, GRO, tissue factor, PDGF (A and B), TGF-β1, cyclooxygenase (COX)-2 and endothelial NOS. It has been recently established that shear stress is capable of activating the MAP kinase signal transduction pathway in vascular endothelial cells (47). Considerable progress has also been made in defining some of the molecular mechanisms involved, including the identification of positive and negative shear stress responsive elements (SSREs) in the promoters of biomechanically responsive genes. (Table. 2.1)

Shear stress responsive genes.				
Gene	Promoter Element	Transcription factor (S)	Pattern of Regulation	Ref
PDGF-B	5'-GAGACC-3'	NF-κB 9p50-p65)	Transient Upregulation	(48, 49)
PDGF-A	Egr-1 sites	Egr-1	Transient Upregulation	(50)
MCP-1	TRE(AP-1) site	AP-1 (c-*fos*, c-*jun*)	Transient Upregulation	(51)
Tissue factor	Sp 1 sites	Sp1	Transient Upregulation	(52)
TGF-β	ND	ND	Transient Upregulation	(53)
ICAM-1	ND	ND	Transient Upregulation	(54)
VCAM1	ND	ND	Downregulation	(55)
Prepro-endothelin-1	ND	ND	Downregulation	(56)

* Biomechanical stimulus = uniform laminar shear stress in physiological range; for the genes listed, the putative SSREs have been identified by analysis of mutated promoter-reporter genes in vitro.
ND, not defined

Table 2.1 Shear stress responsive genes (43)

It appears that the endothelial cell is capable of responding not only to the magnitude of applied forces, but also their temporal and spatial fluctuations – steady versus pulsatile flow, uniform laminar, disturbed laminar or turbulent flow regimes. Most studies to date have used relatively simple *in vitro* fluid mechanic systems to generate uniform laminar shear stresses on cultured endothelial monolayers. In attempts to better model *in vivo* haemodynamics, investigators have devised unsteady flow systems to generate these fluctuations in shear stress and have even combined shear stress, pressure and circumferential stretch in the same *in vitro* systems (54, 57).

Pathophysiological relevance of endothelial activation by fluid shear stress

The most striking example of flow-related changes in vessel wall biology is provided by nature – the non-random distribution of early atherosclerotic lesions in humans and experimental animals. Lesion-prone areas are characteristically those where disturbed flow patterns (flow separation, flow reversal, fluctuating shear stresses) occur such as arterial bifurcations, curvatures and ostial openings. Lesion-protected areas, on the other hand, generally experience uniform laminar flow and relatively constant shear stress such as the straight tubular part of the aorta (43). These patterns also occur in animal models of atherogenesis in whom systemic risks factors such as markedly elevated lipoprotein levels have been deliberately induced. These observations led to speculation that endothelial cells in these lesion-prone areas might be responding differentially to their fluid mechanical environment.

Experiments using RT-PCR-based differential display technology examined the patterns of genes regulated (positively and negatively) by various shear stresses (58). Uniform LSS (as in lesion-protected areas) selectively induces the sustained up-regulation of such genes as manganese superoxide dismutase, COX-2 and NOS. It has been speculated that the antioxidant, anti-thrombotic and anti-adhesive activities of these genes are potentially atheroprotective. Turbulent shear stress (TSS), in contrast, does not induce these genes. Other genes, such as ICAM-1 and MCP-1, do not exhibit *sustained* LSS-selective upregulation. Counter-intuitive to this theory was the finding that TSS (all be it short term for 6 hours) did not result in the up-regulation of genes which are potentially pro-inflammatory/proatherogenic such as ICAM-1 or MCP-1. In another study however the influence of three different flow profiles was investigated – ramp flow (shear stress smoothly transited at flow onset),

step flow (shear stress abruptly applied at onset), and impulse flow (shear stress abruptly applied for 3s only) (59). Impulse flow and the onset of step flow (i.e. temporal gradients in shear) produces marked increases in MCP-1 and PDGF-A expression, while ramp flow and the steady component of ramp flow (steady shear) diminishes their expression, implying a role for temporal gradients in shear in atherogenesis. In addition, further work has demonstrated that oscillatory shear stress, as opposed to static shear stress, resulted in a more sustained activation of pro-oxidative processes as indicated by increased levels of NADH oxidase activity and induction of the redox-sensitive gene heme-oxygenase-1 (60). These authors suggest that differences in the endothelial redox state, orchestrated by different regimens of shear stress, may contribute to the focal nature of atherosclerosis. Finally the recent development of a disturbed laminar shear stress model, which incorporates regions of significant spatial shear stress gradients similar to those found in atherosclerosis prone arterial geometries *in vivo*, has demonstrated increases in levels of nuclear localised NF-κB, Egr-1, c-Jun and c-Fos, as compared with cells exposed to uniform LSS (54). In addition, individual cells demonstrated marked heterogeneity in their responsiveness to disturbed flow as measured by the amounts of these transcription factors in their nuclei. These results imply a role for spatial shear stress gradients in the modulation of endothelial cell gene expression at anatomic sites predisposed to atherosclerotic development.

In summary then, the effects of fluid shear stress to date have been derived from relatively simplified *in vitro* model systems attempting to mimic the *in vivo* setting. It has become apparent that complex flow patterns exist and that it is the nature of these flow profiles in terms of laminar or turbulent flow and their spatial and temporal

fluctuations which may be the determining feature in altering endothelial gene expression.

2.2.2. Transmural hydrostatic pressure

The total transmural hydrostatic pressure to which endothelial cells are exposed to *in vivo* is complex, and is not the same as "blood pressure" as measured with a sphygmomanometer. The hydrostatic pressure, as reviewed by Stronberg and Weiderhielm, is the net sum of several pressures including the vascular hydrostatic pressure (i.e. the force containing blood within the vessels), components of the vascular hydrodynamic pressure ("blood pressure"), plasma and tissue oncotic pressures and the tissue hydrostatic pressure (61). Within a given vascular bed, the hydrostatic pressure is directly correlated with, but generally lower than, arterial blood pressure. A change in blood pressure exposes endothelial cells to changes in hydrostatic pressure. Of the various haemodynamic forces, this is the least investigated. One model of hydrostatic pressure involves growing cells in pressure chambers consisting of cylinders to which medium is then added to attain the desired hydrostatic pressure head. Endothelial cells in one such study were exposed to 1.5-15cm H_2O sustained hydrostatic pressure for up to seven days and exhibited elongation, cytoskeletal reaarangements, increased cell proliferation and bilayering (62). This was accompanied by endothelial release of basic fibroblast growth factor (bFGF). A further study (using the same model) demonstrated increased cell proliferation and increases in α_v integrin expression and altered distribution of focal adhesion plaques (63).

In a different model static or pulsatile pressure was applied to the cells cultured on rigid substrates from above their medium by delivering a mixture of compressed air/CO_2 into specially designed pressure chambers. Higher pressures were used in this study and while endothelial cells demonstrated no gross morphological changes, their proliferation rates decreased (64). The significance of these findings and the intra-cellular signalling pathways underlying them are still uncertain.

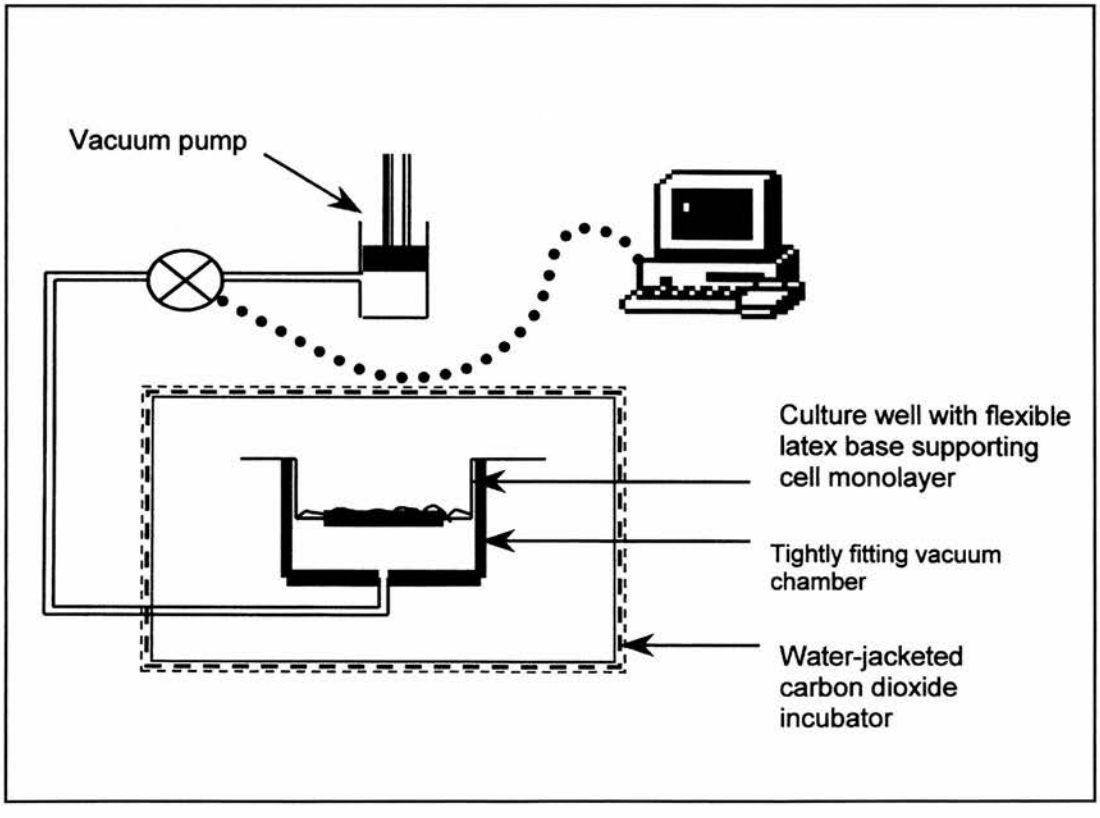
2.3. Mechanical strain: a trigger for vascular cell activation in systemic hypertension

Cyclic strain (also known as mechanical stress/mechanical stretch) describes the elongation and circumferential stresses experienced by cells as the blood vessel rhythmically distends. *In vivo* measurements in patients and animals and *in vitro* models simulating the major geometric features of blood vessels indicate that there is a 5% to 6% wall excursion at peak systole under normal physiological conditions, which can be as much as 10% under hypertensive conditions (65, 66). In addition to increased mechanical strain occurring in the setting of hypertension, recent observations have shown that in the arterial tree, this force is significantly higher at bifurcations and curvatures than in straight segments (67). These considerations suggest that cyclic mechanical stretch may play an important role in atherogenesis. The *in vitro* model most widely used to examine the effects of cyclic strain is the Flexercell unit.

2.3.1. The Flexercell unit

The Flexercell unit (Flexcell International Corporation, McKeesport, PA, USA) is a modification of a unit originally described by Banes et al (68). and consists of a manifold and gasket with eight vacuum ports to receive eight 6 well plates. The culture plates are centred over the vacuum ports, one per port. The strain unit has a fitting to which a vacuum hose is attached. Valves allow adjustment of the ingress and egress of atmosphere and therefore control the degree of elongation of the culture plates. The vacuum hose is connected to a control module which contains pressure transducers, solenoid valves and signal processors and is computer controlled. The computer controls the duration and frequency of the applied stress or

relaxation, while the control module regulates the maximum deformation and strain rates. This allows changes to be made in any of these parameters while holding other variables constant. Cells are grown on specially manufactured culture plates (FlexerCell, McKeesport, PA, USA) that have bottoms made of a flexible silicone rubber material which is then coated with various compounds (collagen, elastin, negatively charged carboxyl coating or positively charged amino hydrophilic coating) depending on the growth requirements of the cells being studied. These bottoms have been tested with stretching up to 200%. Cells therefore on the inner surface of the culture dish experience the same force as applied to the silicone rubber bottoms and real-time video experiments substantiate that the cells remain attached during the cyclic stretching procedure. Control cells are grown in static, but otherwise identical, conditions by culture on non-flexible bottomed coated plates. The various components of this apparatus are shown below (Fig 2.4-The Flexercell).



Several investigators have used this system to assess the effects of mechanical strain on cultured cells. Various stretch regimens have been used. In endothelial studies pressures of between –5 kPa and –20 kPa are generally applied to the underside of the membrane at a frequency of 60 cycles/min (0.5 s of deformation alternating with 0.5 s of relaxation). It is important to point that the degree of stretch across the plate is non-uniform and is maximum at 9.5 mm from the centre of a 25 mm diameter well, decreasing exponentially towards the centre and periphery of the well. –20 kPa results in a deformation ranging from 0% at the centre to a maximum of 24% at the periphery, -5 kPa results in a deformation ranging from 0% in the centre to a maximum of 6% in the periphery. In view of this some investigators have collected cells at the centre and periphery of the wells separately for subsequent analyses.

In vitro studies employing such models of cyclic strain have provided important insights into disease pathogenesis in two major areas. Firstly the exposure of vascular cells, mainly endothelial but also vascular smooth muscle cells, to pathogenic cyclic strain has helped define how the increases in this biomechanical force in hypertension might contribute to arterial hypertensive vasculopathy and, in particular, atherosclerosis (38). Secondly, many forms of glomerular injury are accompanied by compensatory hypertrophy and hyperfiltration of remaining nephrons, resulting in glomerular hypertension and, ultimately, the production of excess extra ECM components by mesangial cells - glomerulosclerosis. Studies into the influence of excessive cyclic strain on mesangial cell function have provided important mechanistic insights into how glomerular hypertension might result in glomerulosclerosis, as will be discussed later in section 2.6 (69).

2.3.2. Endothelial cells and *in vitro* cyclic strain

Endothelial cells subjected to cyclic strain elongate and align themselves so that their long axis is perpendicular to the strain force (70). In addition these cells demonstrate pseudopods and actin stress fibers not found on their statically cultured counterparts. Cyclic strain also stimulates proliferation of endothelial cells (71).

In addition to these structural adaptations, cyclic strain also stimulates endothelial production of a diverse array of biological mediators, as discussed below. Endothelin-1 secretion from endothelial cells is increased five to six-fold by cyclic strain (72). Experiments demonstrate not only an early response to strain (at 20 minutes), but also a later peak in endothelin-1 production. The latter was found to be blocked following treatment with actinomycin-D, suggesting that de-novo synthesis of ET-1 was responsible. Although these studies in bovine aortic endothelial cells showed an increase in endothelin-1 release in response to cyclic strain, they were not able to confirm an increase in transcription of endothelin-1 mRNA. By contrast, another group demonstrated that in human umbilical vein endothelial cells (HUVEC), in addition to a two-fold increase in endothelin-1 release, cyclic strain also induced a two-fold increase in endothelin-1 mRNA levels (73). These authors also provided evidence that this up-regulation was PKC-dependent. The reason for this discrepancy is not clear, but it may indicate species dependancy. ET-1 (section 1.1.1) is released *in vivo* from endothelial cells and binds to endothelin receptors on smooth muscle to mediate contraction. Production of ET-1 is enhanced in several experimental rat models of hypertension (10), and while plasma ET-1 levels are generally not elevated in essential hypertension, there may be an increase in its local concentrations. Endothelin-dependent vascular tone has been demonstrated in studies

of forearm blood flow and enhanced expression of ET-1 mRNA has been shown in the endothelium of small arteries of patients with moderate to severe hypertension (11). ET-1, in addition to causing vasoconstriction, may also function as a co-mitogen for VSMC *in vivo* (21). Given these observations, it is possible that increased haemodynamic forces and cyclic strain, occuring in the setting of hypertension, potentiate endothelin-1 release from endothelial cells. Subsequent ET-1 induced vasoconstriction could theoretically result in further elevation of blood pressure while mitogenic actions on VSMC could promote hypertensive vasculopathy.

In vitro cyclic strain also causes a sustained increase in the expression of the chemokine monocyte chemotactic protein (MCP)-1 in endothelial cells (74). This is accompanied by increases in MCP-1 secretion and a corresponding increase in monocyte chemotactic activity (as measured by monocyte migration assays). Inhibitor studies suggest that the strain-induced MCP-1 expression is predominantly mediated by the PKC pathway and that gene induction requires Ca^{2+}, but is not dependent on cytoskeletal reorganisation. MCP-1 is a potent chemoattractant for monocytes/macrophages and evidence for its role for the recruitment of macrophages into the vessel wall in atherosclerosis has been discussed in detail in section 1.3.1. It has been speculated therefore that strain-induced MCP-1 production might be relevant in hypertension and its contribution to atherosclerosis.

Endothelial activation in response to humoral factors such as cytokines is an established paradigm by which increases in endothelial cell adhesion molecule expression results in increased leukocyte-endothelial adhesion. It is now apparent

that increases in biomechanical stimuli such as shear stress or cyclic strain can also alter adhesion molecule expression. Cyclic strain in endothelial cells increases mRNA levels for ICAM-1 and the release of soluble ICAM-1 from cells (75). Using flow cytometric analysis, increases in ICAM-1 surface expression are both strain and time-dependent and correlated with strain dependent increases in adhesion of monocytes to strained cells. This increase in monocyte adhesion could be partially inhibited by pre-treatment of cells with antibody to ICAM-1. Endothelial expression of E-selectin also increases in response to physiologically relevant levels of cyclic strain, and again, strain-induced monocyte adhesion is substantially inhibited by pre-treatment with anti-E-selectin antibody (76). In contrast, levels of VCAM-1 expression are unchanged in stretched cells over a 24 hour period. As discussed in section 1.3.1, P-selectin mediated adhesion of monocytes to endothelial cells has been implicated both in the early development of atheroma (fatty streak formation) and in ongoing atherosclerosis. In animal models of atherosclerosis endothelial ICAM-1 expression occurs in regions predisposed to atheroma formation as well as on endothelial cells in the atheroscelerotic plaque. Strain-induced expression of endothelial adhesion molecules may contribute to the adhesion of monocytes to localised areas of vascular walls where strain is high (bifurctations, ostia) and therefore to the initiation of atherogenesis. Furthermore, increases in such forces in hypertension may, in part, explain the well-documented association between hypertension and atherosclerosis.

Cyclic strain, like shear stress, increases PDGF-B expression in endothelial cells in a time and amplitude-dependent manner (77). This strain-amplitude dependence was supported by enhanced PDGF-B immunostaining in the high-strain membrane

preriphery relative to cells in the low-strain centre region. The mechanism underlying this up-regulation is still unknown. PDGF (section 2.1.3), a heterodimeric protein composed of two distinct polypeptide chains (PDGF-A and PDGF-B), is a potent smooth muscle cell mitogen. The recent finding of increased plasma levels of PDGF-AB and PDGF-BB in patients with never-treated mild hypertension suggests a role for PDGF in the vascular changes associated with this process (19). Furthermore, PDGF-B gene transcript levels are elevated in human atherosclerotic lesions compared to normal arteries (78). The observation therefore that cyclic strain produces increases in PDGF-B gene and protein expression provides further possible mechanisms for the aetiology of smooth muscle hypertrophy and hyperplasia in hypertension and hypertension-induced atherosclerosis.

Oxidative stress (section 1.3.1) plays an important role in atherosclerosis and is mediated by a variety of reactive oxygen species (ROS) such as superoxide, H_20_2 and hydroxyl radical. The origin of these intracellular ROS remains unclear. They may derive from several sources, including the mitochondrial electron transport system and NADPH oxidase. ROS appear to participate in inflammatory responses by activating transcription factors such as NF-κB which can then induce transcription of a variety of redox sensitive genes such as MCP-1 and ICAM-1. Cyclic strain induces an oxidative stress in endothelial cells as measured by H_20_2 production, release of lipid peroxidation products and activity of NADH/NADPH oxidase (79). Continuous non-cyclic stretching did not induce an oxidative stress suggesting that the pulsatile component of strain is an important feature of the oxidative stimulus. Further studies have suggested that the increases in MCP-1 and ICAM-1 mRNA/protein levels occuring in response to cyclic strain are mediated by strain-induced increases in ROS

(80, 81). In the case of MCP-1, ROS modify the AP-1 binding site in the MCP-1 promoter region, while for ICAM-1 the exact mechanism has yet to be established. Early growth response-1 (Egr-1), a transcription factor and an immediate early gene, is an important regulator for gene expression by competing with the transcription factor Sp-1 in binding to the GC-rich region in the promoter of several atherosclerosis associated genes, including tissue factor, PDGF and ICAM-1 (82). Application of cyclic strain to bovine endothelial cells induces Egr-1 expression prominantly via the Ras/Raf/ERK pathway and strain-induced ROS are involved in the modulation of this signalling pathway (83).

The anti-coagulant and fibrinolytic activity of the endothelium has been previously discussed in section 1.1.1. Cyclic strain results in an increase in endothelial tissue plasminogen activator (tPA) mRNA, immunoreactive tPA protein and tPA activity in the supernatent (84). This seems to occur through the activation of multiple intracellular signalling pathways, including PKA and PKC. These in turn result in the binding of different nuclear factors to the tPA promoter (AP-2 and CRE-like) to initiate new tPA transcripts. Endothelial release of plasminogen activator inhibitor (PAI)-1 also occurs in response to cyclic strain and appears to involve ROS (85). Small areas of denudation and thrombus formation are a common finding on the surface of atheromatous plaques and are usually sub-clinical. In the presence of an imbalance in the coagulation or fibrinolytic systems, such microthrombi may propagate leading to arterial occlusion. TPA initiates fibrinolysis and, in the scenario outlined above, is essential to the dissolution of thombi. PAI-1, on the other hand, inhibits fibrinolysis by forming a complex with tPA and inhibiting free tPA activity. The significance of cyclic strain-induced increases in both these factors is as yet

unclear, but obviously any potential alterations in the endogenous balance of tPA/PAI-1 induced by cyclic strain could have profound implications for thrombosis versus fibrinolysis.

Endothelium-derived nitric oxide (NO) is a potent endogenous vasorelaxant with anti-platelet and anti-leukocyte properties (section 1.1.1). Synthesis of NO in endothelial cells is by nitric oxide synthase (ecNOS). Cyclic strain upregulates the expression of ecNOS transcripts and protein levels in endothelial cells in a strain-dependent manner (86). In cells subjected to 10% cyclic strain increased expression of the ecNOS gene occurred rapidly, 15 minutes after commencement of strain. The mechanisms underlying the regulation of ecNOS by cyclic strain are as yet unknown. Recent studies have demonstrated that sustained dynamic exercise reduces the incidence of cardiovascular events, lowers blood pressure and improves exercise tolerance in patients with ischaemic heart disease. Chronic exercise in dogs increases the release of nitric oxide from large coronary arteries and microvessels and specifically increases ecNOS expression in aortic endothelial cell extracts (87). In humans sustained exercise leads to increased urinary nitrate excretion which indicates an increased NO production (88). It has been speculated therefore that increases in the various biomechanical forces during exercise, including rhythmic distension of the arterial wall, may play a role in the regulation of ecNOS and, ultimately, NO *in vivo*.

An earlier study examined the endothelial production of the vasodilator PGI_2 in response to cyclic strain. It appears that while cyclic strain does not increase basal production of PGI_2 by bovine aortic endothelial cells, it does enhance their ability to

produce PGI_2 when exogenous arachidonic acid (AA) is added (89). TXA_2 production was similar in static and stretched groups in the presence or absence of AA, suggesting that it may be the activity of PGI_2 synthase that is regulated by the repetitive deformation stimulus. The authors of this study conceded that there was marked variability of PGI_2 production by endothelial cells depending on their species, site of origin, passage and confluency. In addition, a deformation regime of 3 cycles/minute was used in this study (as opposed to the more widely used and, in the case of the endothelial cell, more physiologically relevant, 60 cycles/minute), so it is difficult to explain the *in vivo* significance of these results or indeed compare them with other studies.

2.3.3. Mechanical strain to functional response: putative signal transduction mechanisms

Two alternative theories attempt to explain the process of transduction of mechanical strain to biochemical signal (reviewed in (73) and references contained therein). The first paradigm proposes that mechanical strain sensed by cell surface adhesion plaques and cytoskeletal proteins, could mobilise a second-messenger cascade. The second theory relates to the observation that stretch can activate mechanosensitive ion channels in the cell membrane. Potassium, calcium and possibly other ion fluxes across these channels could potentially activate second-messenger signal cascades. Multiple second messenger pathways have been implicated in the transduction of cyclic strain in endothelial cells, as discussed in preceding sections; these include protein kinase C, protein kinase A, calcium, ROS, the Ras/Raf/ERK and the p21ras/phosphatidylinositol-3 kinase/ERK pathways.

Focal adhesion plaques and the cytoskeleton

Mechanical forces will exert their greatest effect at the points of attachment of the cell to the underlying extracellular matrix (ECM). These sites of attachment are known as focal adhesion plaques. They comprise cell surface associated integrin receptors and specific proteins such as paxillin, talin, moesin and focal adhesion kinase (FAK) which mechanically couple the integrins to the cytoskeleton (Fig 2.5a).

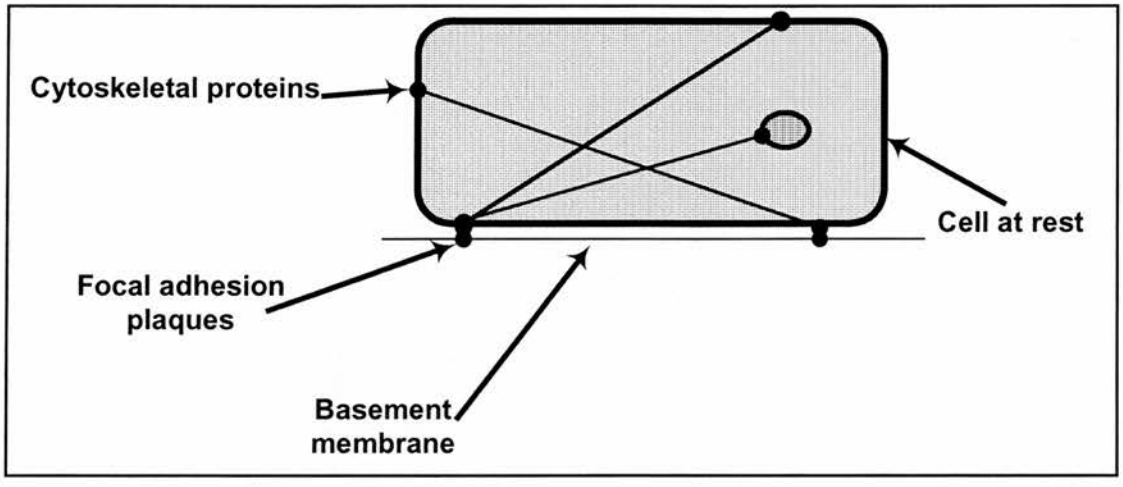


Figure 2.5a Cell at rest: focal adhesion plaques and cytoskeleton

Cytoskeletal proteins including actin, myosin and actinin are tethered to focal adhesion plaques and project from them to span the cell cytosol, inserting into distal aspects of the cell or nuclear membrane. These microfilament bundles experience stretch and deformation in response to external mechanical strain. In short, the cytoskeletal network together with focal adhesion plaques and integrins, form a mechanism, "hard-wired" into cellular architecture, which facilitates the transmission of external physical forces to the internal cell environment including the nucleus (Fig. 2.5b(see over).

Independent groups have highlighted the significance of this pathway. In an intricate series of experiments, Maniotis et al. has demonstrated that when endothelial cell integrins are physically deformed using micropipette techniques, cytoskeletal filaments reorientate, nuclei distort and nucleoli redistribute along the axis of the applied tension (90). These effects are mediated by direct linkages between the cytoskeleton and the nucleus.

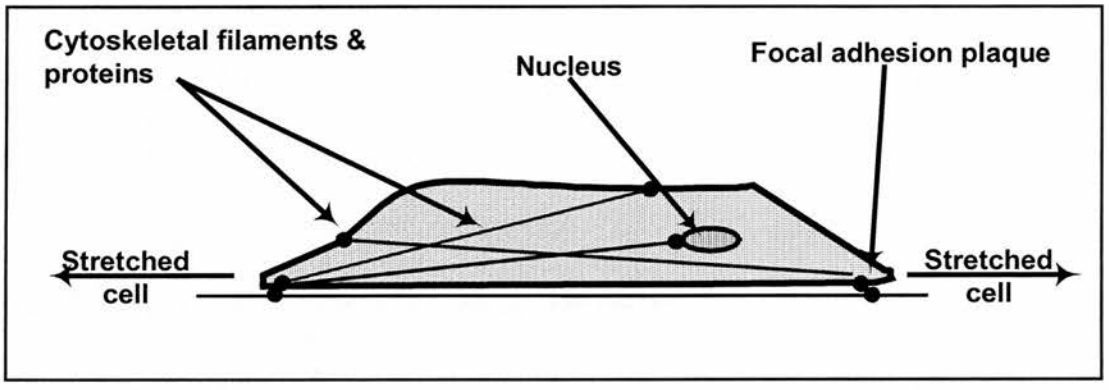


Figure 2.5b Stretched cell: focal adhesion plaques and cytoskeleton

These workers have also reported discovered that cell binding to ECM can result in relocation of mRNA and ribosomes to the focal adhesion plaques (91). These findings, they suggest, demonstrate for the first time, a new mechanism by which integrins may mediate gene regulation. Endothelial cells subjected to periods of cyclical mechanical strain demonstrate increased tyrosine phosphorylation of FAK, paxillin and the small GTP-binding protein rho, as well as a redistribution of FAK and paxillin within the cell. Tyrosine kinase inhibition resulted in loss of the normal pattern of strain-induced EC morphological change and migration, implicating this phosphorylation in the cellular response to mechanical strain (92, 93).

The effects of stretch on ion channels

The existence of specific stretch-activated (SA) cation channels was first proposed in the context of cerebral blood flow autoregulation (94). The channels were described in cerebrovascular smooth muscle cells and were noted for their resistance to the effects of recognised calcium channel blocking agents (95). Since then SA cation channels have been described in several cell types including endothelial and mesangial cells (96, 97). Influx of calcium through stretch-mediated channels has been implicated as the main source for the raised intracellular calcium levels reported with cyclical stretch/relaxation. However a novel stretch activated intracellular calcium store in endothelial cells has recently been identified which may also contribute to the elevated calcium levels (98).

It may be that the ion flux and cytoskeletal based paradigms are not mutually exclusive alternatives. The correct paradigm is likely to contain elements of both.

2.3.4. Regulation of transcription factor activation and gene expression by mechanical forces

Cyclic strain in human umbilical vein endothelial cells (HUVECs) results in induction of the immediate early genes *c-jun, c-fos* and, as already discussed, *Egr*-1. Activation of transcription factors NFκB and AP-1 has also been demonstrated to occur in response to cyclic strain in human endothelial cells (73). While these results confirm transcription factor activation and gene induction by mechanical strain, they do not directly link cyclic strain with gene promotor activation. A common sequence (GAGACC) in the promoter region of platelet-derived growth factor (PDGF) is required for shear stress- induced upregulation of this gene in endothelial cells. This

consensus sequence has been named the Shear Stress Response Element (SSRE) (48). Originally it was reported that activation of the SSRE in stretched endothelial cells occurred within 30 minutes of commencement of 10% cyclical strain. However subsequent studies have indicated that the SSRE was not necessary for inducible gene expression in strained endothelial cells. Work is ongoing to determine if there is a consensus sequence in promoter regions of strain-sensitive genes that specifically modulates the cyclic strain effects in endothelial cells.

2.4. Influence of mechanical strain on cytokine-adhesion molecule networks in ECV-304 cells

2.4.1. Introduction

There is increasing evidence that biomechanical forces can directly influence endothelial structure and function, thus constituting a novel antigen-independent paradigm of endothelial activation (section 2.2, 2.3). The main haemodynamic forces acting on the vasculature *in vivo* include shear stress, transmural (hydrostatic) pressure and cyclic mechanical strain. In hypertension, the latter two forces are increased and result in increased vascular wall stretch. These forces, by inducing expression of a variety of growth-stimulating and pro-inflammatory gene products, could potentially mediate both the altered vessel growth and the pro-atherogenic effects characteristic of this disease. The inflammatory axis of atherosclerosis is discussed in detail in section 1.3.1; in brief, the normally intact and anti-thrombotic endothelium undergoes injury/activation following which monocyte recruitment into the vessel wall occurs via the classical paradigm of leukocyte trafficking. It is hypothesised that increases in cyclic strain, occuring in association with hypertension, may facilitate monocyte recruitment into the vessel wall by acting as a pro-inflammatory stimulus capable of modulating immune mediator expression. The purpose of the current investigation was, therefore, to evaluate whether cytokine, chemokine and adhesion molecule expression in endothelial cells was modified by cyclic strain.

2.4.2. Materials and methods

Cell culture

ECV 304 cells have been previously characterised and were obtained from the EATCC (no. 92091712) (99). All cell culture reagents were obtained from Gibco Life Technologies (Paisley, Scotland) unless otherwise stated. These cells were maintained in M199 medium (Sigma, Dorset, England), supplemented with 1% glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% heat-inactivated foetal calf serum. As the effects of mechanical strain on endothelial cells have been previously shown to be serum-independent, cells were not rendered quiescent prior to exposure to cyclic mechanical stretch.

Mechanical Stretch

ECV 304 cells were seeded at a density of $2x10^5$/well onto elastin-coated, flexible and rigid based six-well plates, (Flex I and II plates respectively, FlexerCell, McKeesport, PA, USA). Vacuum pressure was applied to the underside of these supports by the FlexerCell unit (FX-2000) described in detail in section 2.3.1. Cells were subjected to repetitive deformation with –15 kPa vacuum, i.e. maximum strain 17%, at 60 cycles/min for 24 and 48 hours.

RT-PCR

ECV 304 total RNA was isolated using Trizol reagent (Gibco Life Technologies, Paisley, Scotland) according to the manufacturers' instructions. Total RNA (2μg) was treated with DNase I and $Oligo(dt)_{12-18}$ was then used to generate first strand cDNA. Reverse transcription was carried out using Superscript II RNase H (all from Gibco). Sequence specific primer pairs were either obtained from Biosource (Camarillo,

CA), Continental Laboratories Products Incorporated (CLP, San Diego, CA) or designed and obtained from Sigma-Genoys (Cambridge, UK). (Table 2.2)

Human Gene	Annealing Temp. °C	Product Length, bp	Sequence P1= Primer 1 P2= Primer 2
Adhesion molecules			
ICAM-1	60	237	**P1** 5'-TAT GGC AAC GAC TCC TTC T –3' **P2** 5'-CAT TCA GCG TCA CCT TGG-3'
VCAM-1	60	259	**P1** 5'-ATG ACA TGC TTG AGC CAG G-3' **P2** 5'-GTG TCT CCT TCT TTG ACA CT-3'
E-Selectin	60	435	**P1** 5'-GGG TAG GAA CCC AGA AAC CT-3' **P2** 5'-CCA GAG GAC ATA CAC TGC AT-3'
Chemokines			
MCP-1	60	279	Obtained from BioSource Int. CA, USA
IL-8	60	227	
RANTES	60	197	
Cytokines			
IL-1β	60	331	**P1** 5'-CTT CAT CTT TGA AGA AGA ACC TAT-3' **P2** 5'-AAT TTT TGG GAT CTA CAC TCT CCA-3'
TNF-α	60	325	**P1** 5'-CAG AGG GAA GAG TTC CCC AG-3' **P2** 5'-CCT TGG TCT GGT AGG AGA CG-3'
IFN-γ	60	510	**P1** 5'-ATG AAA TAT ACA AGT TAT ATC TTG GCT TT-3' **P2** 5'-GAT GCT CTT CGA CCT CGA AAC AGC AT-3'
IL-6	60	501	Obtained from BioSource Int. CA, USA
Chemokine Receptors[1]			
CCR1	56	327	**P1** 5'-ACC TGC AGC CTT CAC TTT CCT CAC-3' **P2** 5'-GGC GAT CAC CTC CGT CAC TTG-3'
CCR2	56	255	**P1** 5'-CCA ACT CCT GCC TCC GCT CTA-3' **P2** 5'-CCG CCA AAA TAA CCG ATG TGA TAC-3'
CXCR2	57	385	**P1** 5'-CCG GGC GTG GTG GTG AG-3' **P2** 5'-TCT GCC TTT TGG GTC TTG TGA ATA-3'
CXCR4	60	499	**P1** 5'- TTC CTG CCC ACC ATC TAC TC-3' **P2** 5'-CCA TGA TGT GCT GAA ACT GG-3'
COX[2]			
COX-1	56.5	309	**P1** 5'-CTT GAC CGC TAC CAG TGT GA-3' **P2** 5'-AGA GGG CAG AAT ACG AGT GT-3'
COX-2	55	539	**P1** 5'-AAG CCT TCT CTA ACC TCT CC-3' **P2** 5'-TAA GCA CAT CGC ATA CTC TG-3'
GAPDH			
GAPDH	60	495	**P1** 5'- ACC ACA GTC CAT GCC ATC AC-3' **P2** 5'- TCC ACC ACC CTG TTG CTG TA-3'

1. Reference (100). 2.Reference (101)

Table 2.2 Primer sequences and product sizes

Amplification was as previously described, typically 94^0C for 3 minutes; 35-40 cycles of 94^0C for 30 seconds (denaturing), 55^0C-60^0C for 1 minute (annealing) and 72^0C for 1 minute (extension); followed by a final extension step of 72^0C for 7 minutes. cDNA samples were subjected to parallel PCR reactions with primers for GAPDH to control for equivalency of loading. The presence of genomic DNA was determined by control reactions in which amplification was conducted in complete reaction mixture lacking template cDNA or with RNA samples from RT reactions carried out in the absence of

Superscript II. PCR products were visualised by ethidium bromide staining following electrophoresis on 1.2% agarose gels. Subsequent quantification for each gene studied in comparison to the GAPDH loading control was conducted using the GelWorks 1D software package (UVP, Cambridge UK).

2.4.3. Results and Discussion

In vivo measurements in patients and in *in vitro* animal models simulating the major geometric features of blood vessels indicate that there is a 5% to 6% wall excursion at peak systole under normal physiological conditions, which can extend to 10% in the scenario of hypertension. To determine the effects of pathogenic cyclic strain, cells were stretched at –15 kPa which corresponds to a maximum strain of 17%, (average strain 11%), at a physiologically relevant frequency of 1 Hz (60 cycles/min). While cyclic strain has not been as extensively studied as shear stress, studies to date in endothelial cells have demonstrated the upregulation of a diverse array of genes. These include those for the adhesion molecules ICAM-1 and E-selectin, the chemokine MCP-1, the vasoactive mediator ET-1, the fibrinolytic protein tPA and the growth factor PDGF-B. The implications of these *in vitro* findings, with emphasis on their potential relevance to hypertension-induced atherosclerosis, are discussed in detail in section 2.3.

Human umbilical vein endothelial cells (HUVEC) have been used extensively to study the biology and pathobiology of the human endothelial cell. While the main advantage of HUVEC culture is the wide availability of these cells, there are disadvantages to their use. These include their time-consuming isolation, fastidious growth factor requirements, relatively short life-span and long population doubling

time. These limitations drove a search for transformed endothelial cell lines. In 1990, the establishment of an immortalised HUVEC cell line, ECV-304 was reported (99). A number of groups demonstrated that these cells exhibited classical endothelial properties including the expression of characteristic markers (UEA-1 lectin, PHM5 and HAM 56), organelles (Wiebel-Palade bodies) and the ability to form blood vessels (angiogenesis) (102). It must be pointed out however that the use of a cell line rather than freshly isolated primary cells involves accepting the caveat that the acquisition of the transformed phenotype may alter key cellular characteristics of the former. For example ECV-304 lack vonWillebrand factor and express cytokeratins usually associated with cells of an epithelial origin. Despite these discrepancies, ECV-304 have been employed as a valid model of endothelial cell function by numerous investigators.

In order to validate the current experimental model, the expression of the adhesion molecule ICAM-1 and the chemokine MCP-1 were first examined, since cyclic strain has been reported to modulate endothelial expression of both these genes (74, 75). In the present study, constitutive expression of ICAM-1 in ECV-304 cells was apparent and mRNA levels for this species were increased by exposure to cyclic mechanical stretch for 24 and 48 hours. (Fig 2.5). MCP-1 was also constitutively expressed in these cells but, in contrast to ICAM-1, no upregulation was apparent after 24 hours of cyclic strain. Exposure to mechanical stretch for 48 hours, however, resulted in a marked increase in MCP-1 mRNA expression as compared with control cells which were grown under identical, but static conditions (Fig 2.6). This is in agreement with previous studies and the ability to reproduce these results validated the chosen experimental parameters chosen for the current study.

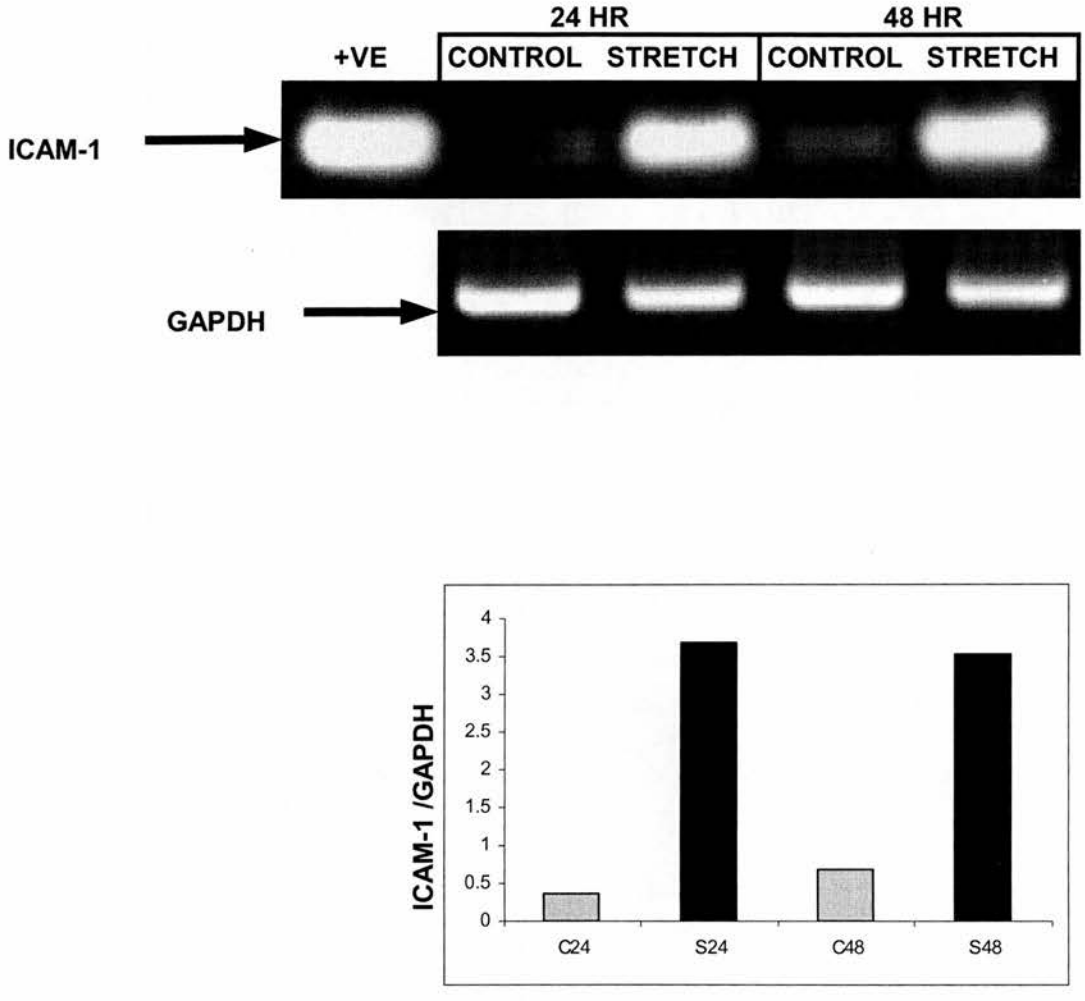


Figure 2.6. ICAM-1expression in ECV-304 cells is upregulated by cyclic strain

Constitutive expression of ICAM-1 is apparent, but mRNA levels for this species are elevated following 24 and 48 hours of cyclic mechanical strain. Positive control is TNF-α treated ECV-304 cells. Result shown is representative of three separate experiments (Appendix 1A).

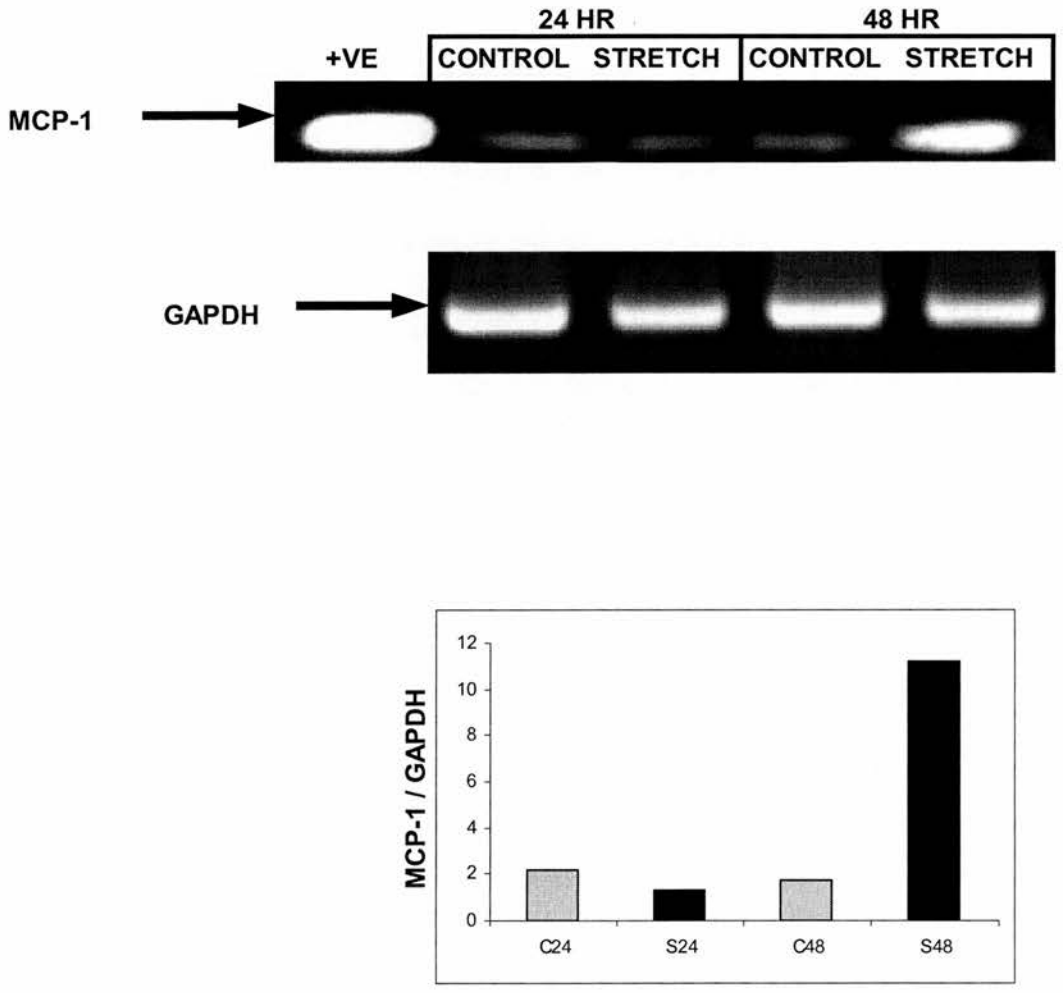


Figure 2.7.MCP-1 expression in ECV-304 cells is increased by cyclic strain

Constitutive expression of MCP-1 is apparent, but mRNA levels for this species are elevated following 48 hours of cyclic mechanical strain. Positive control is TNF-α treated ECV-304 cells. Result shown is representative of three separate experiments (Appendix 1A).

The emerging role of chemokines, and in particular IL-8, in the inflammatory axis of atherogenesis has been discussed previously (section 1.3.1). The expression of IL-8 and its corresponding receptor CXCR-2 has been detected in human atherosclerotic lesions. The absence of the murine homologue of this receptor (mIL-8R) on bone marrow derived-peripheral blood leukocytes in LDL-receptor deficient mice has been reported to result in reduced atherosclerotic lesion size and attenuated macrophage recruitment, implying a role for IL-8 receptor binding chemokines in the localisation of macrophages to these lesions. IL-8 and its related chemokines were originally thought to be predominantly chemotactic for neutrophils which are scanty in human atherosclerotic lesions. Recent work however has suggested that IL-8 may facilitate macrophage recruitment into the atherosclerotic plaque via other, non-chemotactic, mechanisms. It has been demonstrated, for example, that IL-8 is a potent trigger for firm adhesion of monocytes to vascular endothelium expressing cell adhesion molecules under flow conditions; in this study IL-8-induced monocyte arrest was significantly blocked a monoclonal antibody to β2 integrin (103).

These studies implicate IL-8 in monocyte recruitment to the atherosclerotic plaque by translating initial monocyte tethering into firm adhesion through activation of leukocyte integrins. IL-8 has also been recently identified in human coronary atherectomy specimens where its presence has been implicated in angiogenesis as assessed using *in vivo* assays (104). The corneal neovascular response was markedly positive in response to pellets from atherectomy specimens as compared with controls and was completely abrogated by IL-8 neutralising antibody. In addition, IL-8 is a potent chemoattractant and mitogen for VSMC *in vitro* (105). Increased endothelial IL-8 expression in response to mechanical stretch therefore, in addition to

facilitating macrophage influx/adhesion, might also provide a potential mechanism whereby increased cyclic strain, occuring in the setting of hypertension, could mediate the medial smooth muscle hyperplasia characteristic of this disease.

We chose to examine therefore whether cyclic mechanical strain could modulate IL-8 expression in ECV-304 cells. mRNA for IL-8 was expressed under basal conditions in these cells and cyclic strain led to increased expression of this C-X-C chemokine (Fig. 2.8). This was most pronounced following 24 hours of mechanical strain, but was still evident at 48 hours. ECV-304 cells treated with TNF-α at a concentration of 100 ng/ml for 4 hours served as a positive control.

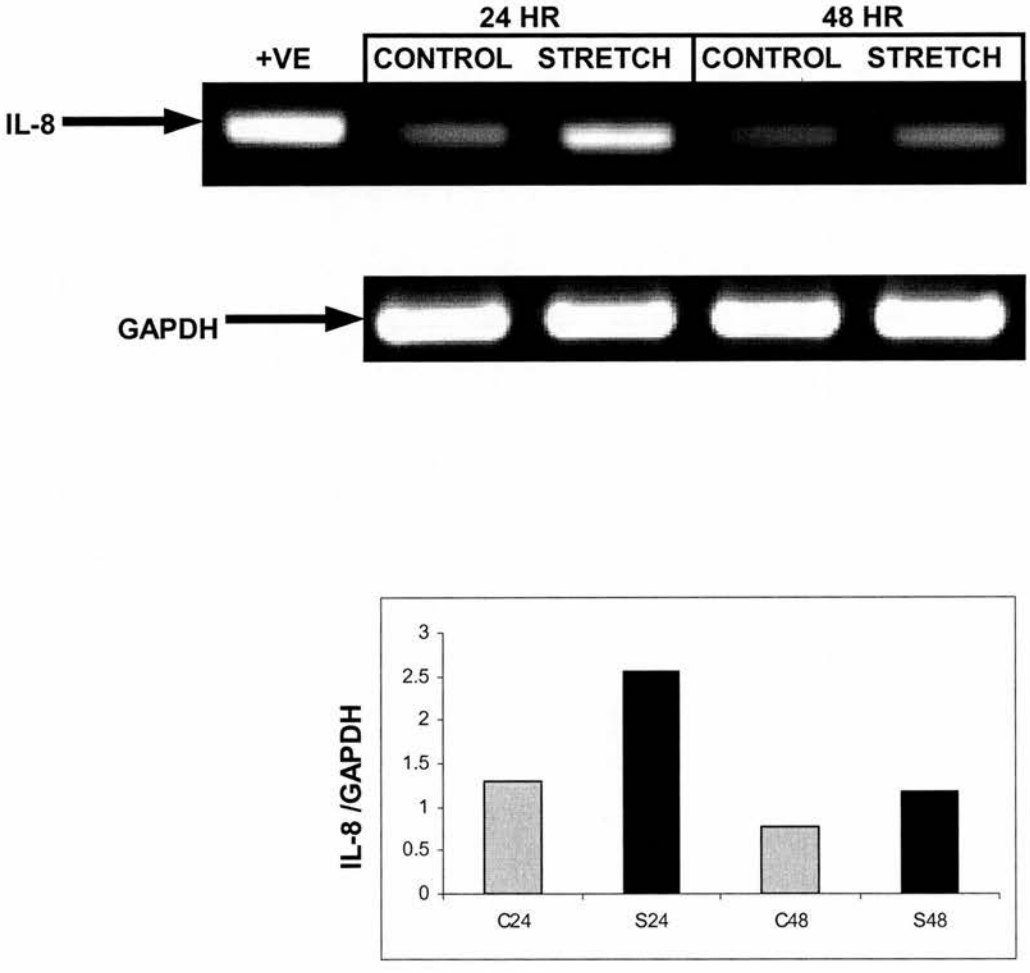


Figure 2.8. IL-8 expression in ECV-304 cells is increased by cyclic strain

Constitutive expression of IL-8 is apparent, but mRNA levels for this species are elevated following 24 and 48 hours of cyclic mechanical strain. Positive control is TNF-α treated ECV-304 cells. Result shown is representative of three separate experiments (Appendix 1A).

*R*egulated upon *a*ctivation, *n*ormal *T* cell *e*xpressed and *s*ecreted (RANTES) is a C-C chemokine that attracts monocytes, T lymphocytes and eosinophils into sites of inflammation (1.2.2). In keeping with this role, RANTES expression has been identified in a wide range of inflammatory diseases including endotoxaemia, inflammatory bowel disease, acute pulmonary disease and rheumatoid arthritis. In one study RANTES expression was found to be elevated in the cells of atherosclerotic lesions of carotid endarterectomy specimens. However, while RANTES expression was identified in a number of atherosclerotic sites (abdominal aorta, iliac and carotid arteries) by other investigators (106), this chemokine was also detected in apparently normal aortic control tissue (100); the role of RANTES in the pathogenesis of atherosclerosis is, therefore, still unclear. RANTES expression can be induced in vascular smooth muscle and endothelial cells *in vitro* in response to pro-inflammatory cytokines (TNF-α, IFN-γ) (107). On the basis that cyclic strain is a putative pro-inflammatory stimulus, the expression of RANTES in ECV-304 cells exposed to cyclic strain was examined. In contrast to IL-8, the expression of RANTES, although constitutive, was not upregulated in ECV-304 cells following 24 or 48 hours of cyclic strain (Fig 2.9). There was, however, a marked upregulation in the expression of this chemokine following stimulation of these cells with TNF-α (100 ng/ml for 4 hours). The observation in the current study that cyclic strain does not modulate RANTES expression, but does increase expression of both IL-8 and MCP-1, demonstrates differential chemokine responses in ECV-304 cells exposed to cyclic strain. The data do not, however, rule out a role for RANTES in hypertension-associated atherosclerosis since its expression may be modulated by additional classic pro-inflammatory stimuli present in the vascular milieu.

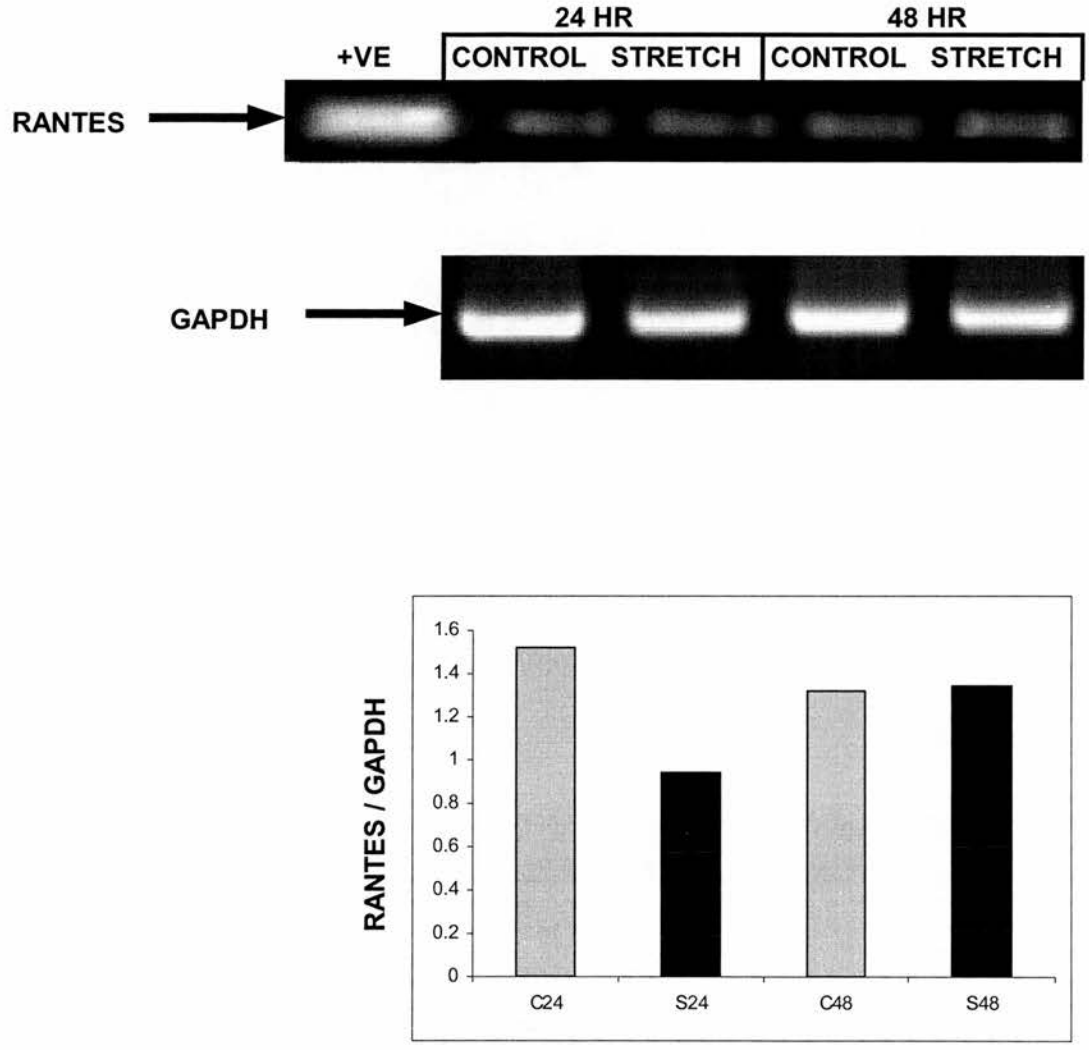


Figure 2.9 RANTES expression in ECV-304 cells is unaltered by cyclic strain

Constitutive expression of RANTES is apparent, but mRNA levels of this chemokine remain unchanged following 24 and 48 hours of cyclic mechanical strain. Positive control is TNF-α treated ECV-304 cells. Result shown is representative of three separate experiments (Appendix 1A).

The biological effects of chemokines are mediated through interactions with their corresponding chemokine receptors which are expressed in a cell-type specific manner (section 1.2.4). Chemokine receptor expression can be modulated by a variety of pro-inflammatory stimuli. Ligands for CCR1 are the C-C chemokines RANTES, MIP-1α, MCP-2 and MCP-3, while MCP-1-4 bind CCR2.

The functional importance of the CCR2 receptor was demonstrated recently when homologous recombination was used to "knock-out" its expression in atherosclerotic prone mice (108). Apolipoprotein E null mice (which develop severe atherosclerosis) deficient in the CCR2 receptor exhibited markedly decreased lesion formation and macrophage recruitment (discussed in section 1.3.1). These results identified CCR2 as a possible genetic determinant of murine atherosclerosis and provided evidence for the role of MCP-1 in macrophage recruitment and, hence, atherogenesis. Conflicting reports exist as to the presence of the C-C chemokine receptors on endothelial cells (section 1.3.1). A number of groups have been unable to detect CCR1 and CCR2 expression; a recent study, however, demonstrated endothelial expression of CCR2, in addition to MCP-1 induced migration of endothelial cells *in vitro*. Similarly, conflicting reports exist as to the expression of the IL-8 receptor on endothelial cells. The presence of this receptor would not be unexpected, given the finding that IL-8 increases endothelial proliferation and migration *in vitro* and promotes angiogenesis *in vivo*, presumably through a receptor-mediated mechanism.

The expression of CXCR4 on endothelial cells has been demonstrated previously (109). The constitutive nature of this receptor and its ligand, stromal derived factor (SDF)-1, led to speculation that together they may play an important role in the early

recruitment of monocytes and T lymphocytes, particularly since inducible chemokines such as MCP-1 are generally not expressed on normal arteries. In addition, the expression of CXCR4 in endothelial cells is modulated by various pro-inflammatory mediators implicated in the pathogenesis of atherosclerosis. IFN-γ for example results in a down-regulation of CXCR4 expression in endothelial cells, while IL-1β and TNF-α produce a biphasic response eliciting an immediate decrease, followed by a subsequent reversal and increase in steady state levels of CXCR4 mRNA (109).

In light of the above findings, *basal* expression of the chemokine receptors CCR1, CCR2, CXCR2 and CXCR4 in ECV 304 cells was investigated. The influence of mechanical strain on receptor expression levels was also examined. Neutrophil and mononuclear cell total RNA served as a positive control for these experiments. CCR1 and CCR2 are expressed by monocyte/macrophages, while CXCR2 is expressed by neutrophils (110). These controls were used in the relevant PCR reactions.

CCR1, CCR2 and CXCR2 were not expressed in ECV-304 cells, nor was mRNA for these species induced by cyclic strain (Fig 2.10). CXCR4, however, was expressed constitutively in ECV-304 cells. Expression of this receptor, as compared to control cells, exhibited a biphasic pattern; at 24 hours expression was decreased while at 48 hours expression was elevated over control (Fig 2.11). Comparisons may be drawn between these observations and the biphasic response of endothelial CXCR4 expression previously demonstrated by other investigators in response to IL-1β and TNF-α. The latter, however, occurs over a different time period, with initial

decreases occuring in the first three to five hours following cytokine stimulation, while increased expression is apparent by 24 hours. Increases in CXCR4 expression in endothelial cells by cyclic strain could be potentially relevant to the *in vivo* recruitment of monocytes and T cells to the vessel wall in hypertension-induced atherosclerosis. However, it is recognised that RT-PCR is a semi-quantitative technique and that these preliminary results must be confirmed using more quantitative methods such as Northern blotting.

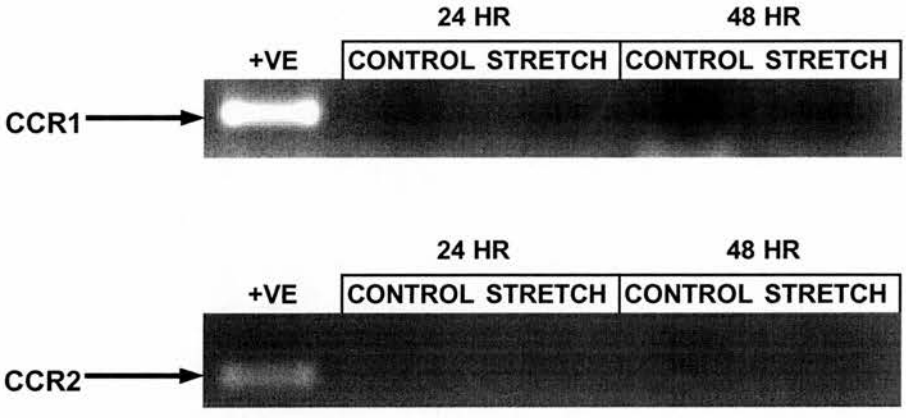


Figure 2.10 Chemokine receptor expression in EVC-304 cells

The chemokine receptors CCR1 and CCR2 are not expressed by ECV-304 cells under basal conditions or in response to cyclic strain. Human monocytes served as a positive control in these experiments. Result shown is representative of two separate experiments

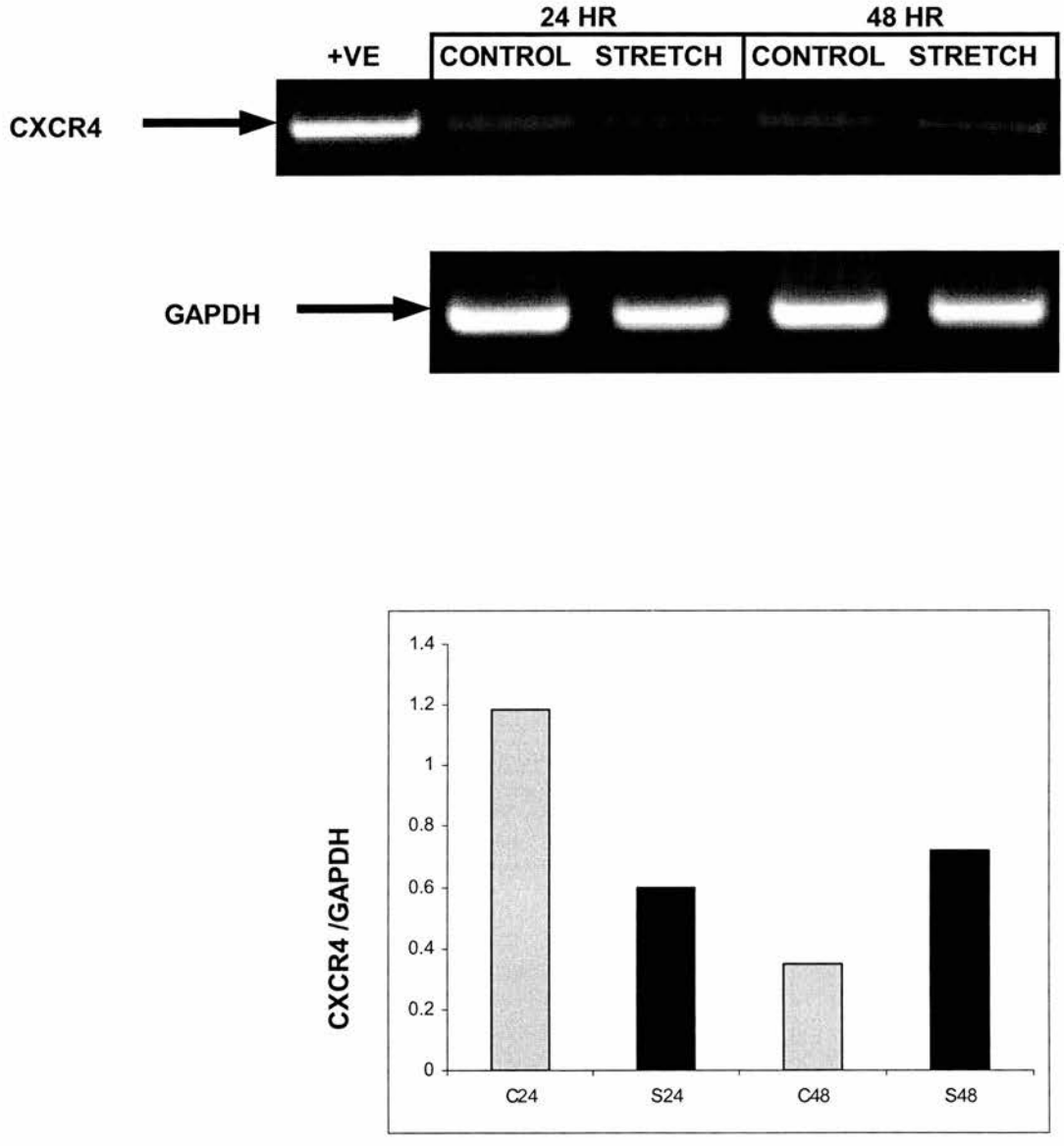


Figure 2.11 Expression of the chemokine receptor CXCR4 in ECV-304 cells is modulated by cyclic strain

The receptor CXCR4 was constitutive in ECV-304 cells. Expression of this receptor, as compared to control cells, appeared to be downregulated and then upregulated following 24 hours and 48 hours of cyclic strain, respectively. Human monocytes served as a positive control for this receptor. Result shown is representative of three separate experiments(Appendix 1A).

While endothelial expression of the adhesion molecules E-selectin and ICAM-1 can be modified by cyclic strain, conflicting reports exist as to modulation of VCAM-1 mRNA levels by cyclic strain. One study demonstrated upregulation of VCAM-1 by approximately 30% following 24 hours of cyclic strain in HUVEC (111) while another group was unable to reproduce this increase (76). Strain-induced expression of such endothelial adhesion molecules *in vivo* may facilitate the adhesion of monocytes to localised areas of vascular walls where strain is high (bifurctations, ostia) and, therefore, to the initiation of atherogenesis. Furthermore, increases in such forces in hypertension may, in part, explain the well-documented association between hypertension and atherosclerosis. Given these observations, it was of interest to us to explore the influence of cyclic mechanical strain on the expression of E-selectin and VCAM-1 in ECV-304 cells. E-selectin was not expressed in these cells under basal conditions or, indeed, in response to 24 or 48 hours of cyclic strain. TNF-α (100 ng/ml for 4 hours) resulted in a slight increase in mRNA levels as detected by RT-PCR. VCAM-1 was not expressed under basal conditions in these cells nor in response to cyclic strain, but once again was detected in TNF-α-treated cells (Fig 2.12).

Growing evidence implicates cytokines, in addition to their chemokine sub-group, in the formation and development of atherosclerotic lesions. Their role in this process, mediated predominantly by their effects on cell proliferation and lipid/protein synthesis, is discussed elsewhere (1.3.1). On the basis that cyclic mechanical strain is a putative pro-inflammatory stimulus, the effects of this force on the expression of the cytokines TNF-α, IL-1, IFN-γ and IL-6 in ECV-304 cells were examined.

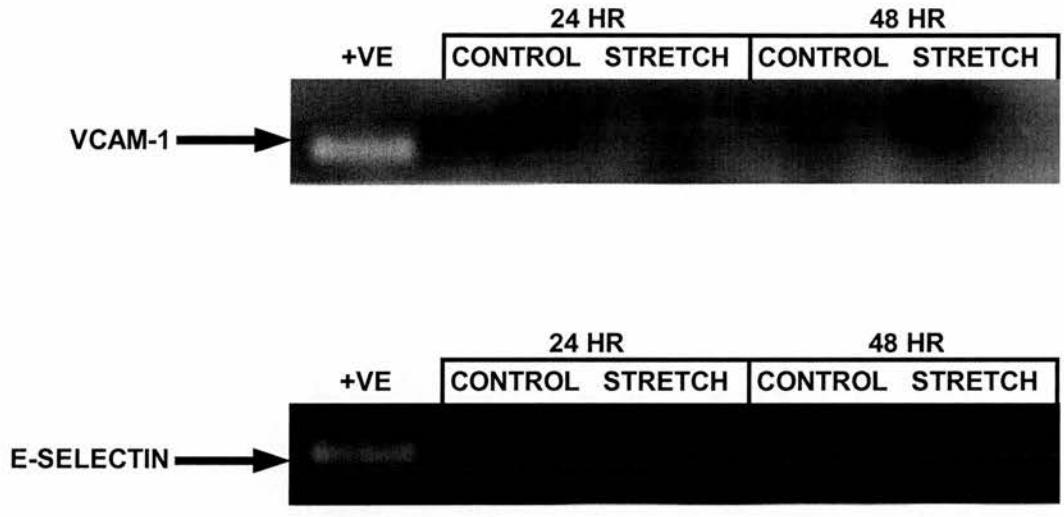


Figure 2.12 VCAM-1 and E-selectin in ECV-304 cells are unaltered by cyclic strain

VCAM-1 and E-selectin are not expressed by ECV-304 cells under basal conditions or following exposure to cyclic strain; in contrast, in TNF-α treated ECV-304 cells, faint expression of these adhesion molecules is detected. Result shown is representative of three separate experiments

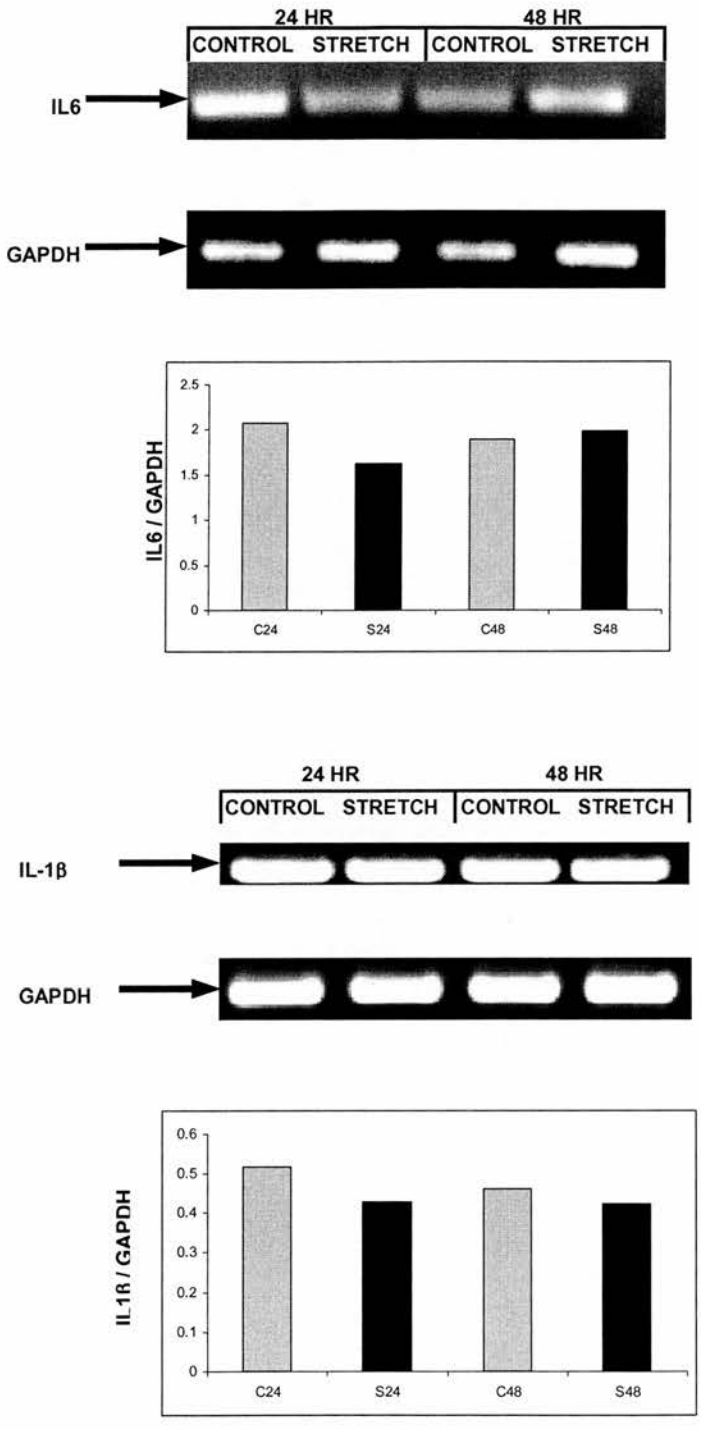


Figure 2.13. Expression of the cytokines IL-6 and IL-1β in EVC-304 cells are unaltered by cyclic strain

IL-6 and IL-1β are expressed in ECV304 cells under basal conditions but mRNA levels are unaltered by cyclic strain. Result shown is representative of two separate experiments.

IFN-γ is not expressed in ECV-304 cells under basal conditions nor in response to cyclic mechanical strain. By contrast, TNF-α, IL-β and IL-6 were found to be expressed constitutively in ECV-304 cells. As was found for IFN-γ however, mRNA levels for the cytokines remain unaltered following 24 and 48 hours of cyclic strain (Fig 2.13).

Cyclooxygenase (COX) catalyses the first, rate-limiting step in the formation of prostaglandin and thromboxane eicosanoids from phospholipase A2-released arachidonic acid. The "inducible" form of the enzyme, COX-2, is absent from most cells under basal conditions, but is rapidly and transiently expressed in response to a diverse range of stimuli. As might have been anticipated from its *in vitro* induction by lipopolysaccharide and pro-inflammatory cytokines, COX-2 has been found to be important in a wide variety of inflammatory disorders; indeed, the development of selective COX-2 inhibitors as therapies for inflammatory disease represents a key area of current pharmacological research (112). A close correlation between the inflammatory response and COX-2 activity is supported by the observation that its expression is induced by inflammatory cytokines such as interferon-γ and tumour necrosis factor-α (113) and downregulated by the anti-inflammatory cytokine IL-10. Further, examination of synovia from patients suffering from osteo- and rheumatoid arthritis has confirmed the presence of COX-2 in mononuclear cells, blood vessel endothelial cells and sub-synovial fibroblast-like cells (114) supporting the contention that the major portion of inflammatory prostaglandins are derived from COX-2. COX-2 "knockout" mice have, however, provided unexpected results in a number of inflammatory assays (115). These studies indicated that inflammatory responses in the carrageenan, phorbol ester and arachidonate models were COX-2

independent and moreover, these animals were observed to exhibit normal inflammatory responses to bacterial invasion of the peritoneum. It has been recently demonstrated *in vitro*, however, that COX-1$^{-/-}$ and COX2$^{-/-}$ cells derived from these animals exhibit substantially enhanced basal and inducible expression of the remaining functional COX gene, in addition to increased expression of cytosolic phospholipase A_2 ($cPLA_2$), another key enzyme in prostaglandin synthesis (116). Such compensatory COX-1 production in the COX2$^{-/-}$ mice almost certainly explains the counter-intuitive findings regarding the inflammatory responses of these animals.

Inducible COX-2 was initially regarded as a pro-inflammatory enzyme as evidenced by its increased expression and bioactivity in disorders such as rheumatoid arthritis. However, evidence is accumulating that in certain scenarios this enzyme may also elicit anti-inflammatory effects (117). In carrageenin-induced pleurisy in rats, COX-2 protein expression in inflammatory exudate cells peaked at 2 hours and again at 48 hours (time points that in this model are associated with predominant PMN and mononuclear cell infiltration, respectively). While the initial peak was associated with maximal PGE_2 synthesis, the second, and more pronounced peak, coincided with minimal PGE_2 production and increased levels of PGD_2 and 15-deoxy-PGJ_2. Furthermore, the selective COX-2 inhibitor NS-398 and the dual COX-1/COX-2 inhibitor indomethacin, while inhibiting inflammation at 2 hours, significantly exacerbated inflammation at 48 hours (as measured by increases in cellular infiltrates and exudative volume). This exacerbation was associated with reduced production of PGD_2 and 15-deoxy-PGJ_2 in exudates and reversed by the exogenous addition of these prostaglandins. On the basis of these observations, it has been suggested in this model COX-2 may be pro-inflammatory during the early PMN-dominated phase, but

may *aid* inflammatory resolution in the later mononuclear cell- dominated stage by generating alternative anti-inflammatory prostaglandins. Similarly in a model of experimental glomerulonephritis COX products (athough in this case predominantly *COX-1*-derived) have been implicated in the endogenous repression of the chemokine MCP-1, an event that coincides with a reduction in glomerular monocytes and resolution of this disease (118).

In the vascular scenario, it appears that COX-2 may also have immunomodulatory effects following vessel injury (reviewed in (119)). In animal vessels *in vivo* and *in vitro* COX-2 expression can be induced in vascular smooth muscle cells in response to physical injury and pro-inflammatory cytokines. Moreover, this upregulation is associated with increases in PGI_2 and PGE_2 production that are able to compensate for the loss of prostaglandin synthesis (via constitutive COX-1) by injured endothelial cells. PGI_2, as discussed in section 1.1.1, is a vasodilator with potent inhibitory actions on platelet function and is essential in the maintenance of normal vascular homeostasis. Studies *in vitro* demonstrate that COX-2, induced in response to cytokines, limits VSMC adhesion molecule expression (ICAM-1 and VCAM-1) and GM-CSF release. On this basis, it has been proposed that the induction of COX-2 in VSMC may represent a defence mechanism compensating for COX-1 loss in the endothelium which, via suppression of cytokine and adhesion molecule expression, limits further vascular damage. The expression of COX-1 and COX-2 in human atherosclerotic lesions has recently been examined (120). While normal arteries demonstrated COX-1 (but not COX-2) mRNA and protein in the endothelial and medial smooth muscle cells, atherosclerotic lesions contained both COX-1 and COX-2 in macrophages, and to a lesser extent in VSMC and endothelium. The role of

COX-2 in the pathogenesis of atherosclerosis has not been definitively established and, indeed, based on current evidence this enzyme could potentially either promote atherosclerosis via its pro-inflammatory effects or be produced in response to pro-atherosclerotic stimuli to limit plaque progression and prevent further vessel injury. To date, it may be only speculated as to whether its effects result in a positive or negative modulation.

On the basis of the hypothesis that cyclic strain is a pro-inflammatory stimulus, the putative influence of this force on the expression of COX-1 and its inducible isoform, COX-2, in ECV-304 cells was examined. COX-1 is expressed in ECV-304 cells under basal conditions, but levels remain unchanged by cyclic strain (Fig. 2.14). In contrast, mRNA for COX-2, while also present in these cells under basal conditions, increases following 48 but not 24 hours of cyclic strain (Fig 2.15). COX-2 is serum-inducible, so, as expected, expression levels in the control cells decline over the time course studied (Fig 2.15). Based on the current available evidence regarding COX-2 bioactivity within the vasculature, it is speculated that the strain-induced upregulation of COX-2 in ECV-304 could potentially modulate the response to this stimulus by influencing the expression of leukocyte trafficking determinants (chemokines and leukocyte adhesion molecules).

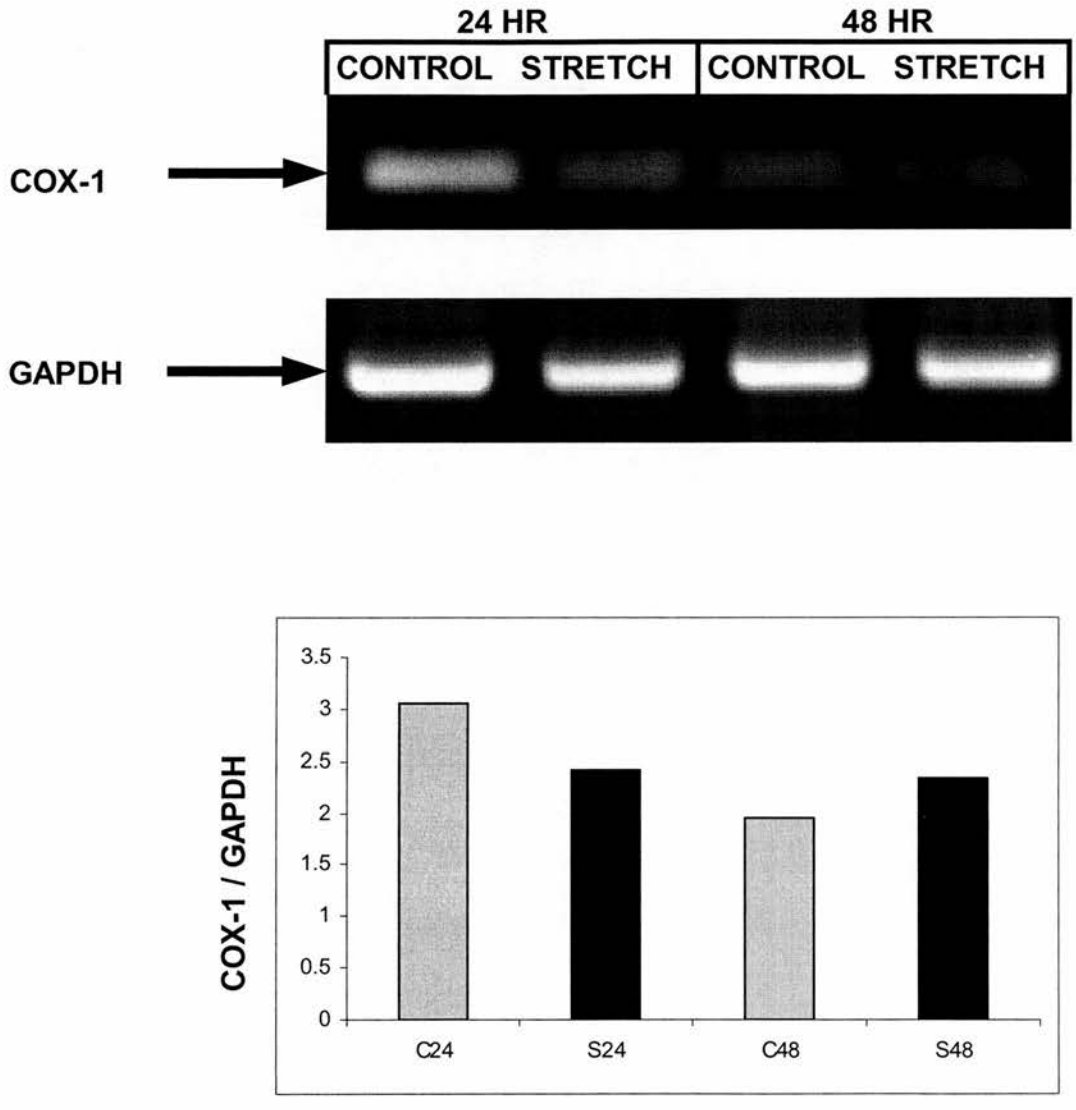


Figure 2.14 COX-1 expression in EVC-304 cells is unaltered by cyclic mechanical strain

COX-1 is expressed in EVC-304 cells under basal conditions, but mRNA levels for this species remain unchanged in response to cyclic strain. Result shown is representative of three separate experiments (Appendix 1A).

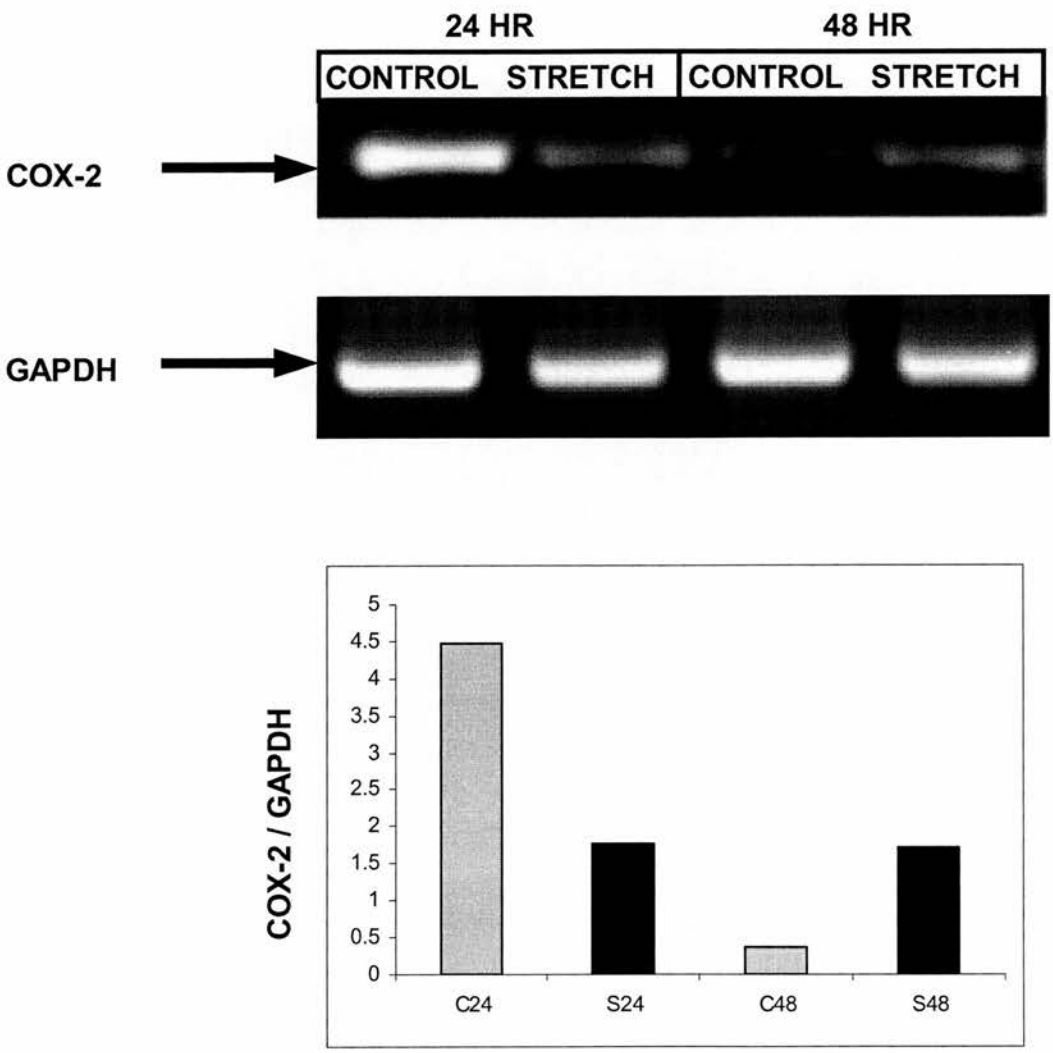


Figure 2.15. COX-2 expression in ECV-304 cells is increased by cyclic strain

mRNA for COX-2 is present in ECV-304 cells under basal conditions. Increases in COX-2 expression occur following 48 but not 24 hours of cyclic strain. COX-2 is serum-inducible, so, as expected, expression levels in the control cells decline over the time course studied. Result shown is representative of three separate experiments (Appendix 1A).

As this work neared completion, results obtained from PCR-based single locus fingerprinting studies questioned the origin of ECV-304 cells. In June 1999 a directive was issued from the European Collection of Cell Cultures (EATCC), from whom these cells had been initially obtained by our laboratory, which suggested that ECV-304 had the same DNA fingerprint as the T24 cell line (human bladder carcinoma (epithelial)).

This discovery has obvious implications for the findings of the current study. For example, our inability to reproduce the previous finding that cyclic strain induced upregulation of E-selectin is most likely explained by the fact that ECV-304 cells are epithelial in origin. The observation in the current study that TNF-α can induce expression of E-selectin mRNA in ECV-304 cells is interesting, since E-selectin has been previously considered to be expressed almost exclusively by activated endothelial cells. A recent study however has identified E-selectin expression in colonic epithelial cells (121).

Irrespective of source however this cell line provides a useful model for the regulation of immune mediators by mechanical strain. Although we cannot speculate on the basis of these results on the pro-atherogenic potential of cyclic strain, a number of interesting observations can still be made. Inadvertently, this study has identified strain-induced genes in epithelial cells. The results described here outlining the modulation of chemokine and adhesion molecule expression by cyclic strain in ECV-304 cells are otherwise in broad agreement with the results described by other groups working with other cell types. The upregulation of ICAM-1 and MCP-1 have

been previously demonstrated in endothelial cells and, indeed, were used in the current study to validate the *in-vitro* conditions. During the course of this work, cyclic strain induced upregulation of IL-8 mRNA in endothelial cells was demonstrated simultaneously by another group (122). IL-8 expression, therefore, in both endothelial and epithelial cells, is increased by cyclic mechanical stretch. Likewise, the observation in the current study that cyclic strain increases expression of COX-2, but not COX-1, in epithelial cells has been previously documented in mesangial cells and bladder smooth muscle cells (123, 124). Furthermore, increases in COX-2 promoter activity in response to cyclic strain have recently been demonstrated in endothelial cells. Investigators elsewhere have demonstrated that cyclic mechanical stretch increases expression of the growth factor PDGF-B in both endothelial cells and vascular smooth muscle cells (in the latter PDGF-A is also upregulated in a strain-dependent manner) (77, 125). In addition, in agreement with the current study, other groups have failed to demonstrate increases in mRNA for the cytokines TNF-α, IL-1 and IL-6 in response to cyclic strain in HUVEC (122). It would appear that distinct cell types share "strain-inducible" genes (Table 2.3).

The observation that specific genes are upregulated in a number of different cell types in response to cyclic mechanical stretch raises the possibility that these responses are, to some degree, a strain-specific phenomenon. A cell specific level of control may also exist, however, since IL-8 expression was found to be negatively modulated by cyclic strain in mesangial cells (section 2.6).

Increased expression in response to cyclic mechanical strain	Cell type
COX-2	Endothelial Epithelial Mesangial
MCP-1	Endothelial Epithelial
ICAM-1	Endothelial Epithelial
IL-8	Endothelial Epithelial
PDGF-β	Endothelial VSMC

Table 2.3 "Strain-inducible" genes common to different cell types.

A number of alternative theories have attempted to explain the process of transduction of mechanical strain to biochemical signal. (Section 2.3.3). One such theory proposes that mechanical strain, sensed by cell surface adhesion plaques and cytoskeletal proteins, could mobilise second-messenger cascades. In support of this theory both endothelial and mesangial cells subjected to periods of cyclical mechanical strain demonstrate increased tyrosine phosphorylation and redistribution of focal adhesion plaque proteins FAK and paxillin (69, 73). Multiple second messenger pathways have also been implicated in the responses of endothelial cells, mesangial cells and vascular smooth muscle cells to mechanical strain. These are discussed in detail elsewhere. In brief, the activation of PKC and PKA, stretch-induced calcium fluxes and mitogen activated protein (MAP) kinases appear to play a role in the strain-induced responses of all three cell types. Downstream to such signalling cascades the induction of various immediate early genes including *c-fos* and *egr-1* occurs following the exposure of these cell types to cyclic strain.

These findings lend further support to the theory of strain-dependent transcription, mediated by a common strain-responsive consensus sequence in the promoter region of target genes. A recent study in vascular smooth muscle cells exposed to cyclic mechanical strain identified a GC-rich region in the proximal 92 bp of the PDGF-A promoter as a putative strain-responsive element (125). These authors presented evidence that this region contains mechanical strain responsive elements that bind the transcription factors Egr-1, and possibly Sp-1. To date, a common consensus sequence in promoter regions of other strain-sensitive genes in any other cell type remains to be identified.

Recently another theory has emerged on how mechanical stimuli are converted into biochemical stimuli which may also explain the presence of strain-responsive genes common to diverse cell types. Workers using vascular smooth muscle cells showed that cyclic strain evoked activation of MAP kinases followed by enhanced DNA binding activity of the transcription factor AP-1 (126). In addition, this force rapidly induced phosphorylation and activation of PDGF receptor-α. Conditioned medium from the vascular smooth muscle cells exposed to mechanical strain did not result in PDGFR-α phosphorylation and antibodies binding to all forms of PDGF did not block strain-induced PDGFR-α activation. These authors speculated that mechanical stresses, by directly perturbing the cell membrane, may alter receptor confirmation, thereby initiating signalling pathways normally activated following ligand-dependent receptor activation. While this has yet to be demonstrated for other cell types or indeed other receptors, in theory, simple deformation of the membrane and activation of receptors in this manner could induce the expression of a variety of mediators, irrespective of the cell type.

The current study demonstrated the upregulation of ICAM-1, MCP-1, IL-8, COX-2 and CXCR4 expression in ECV-304 cells in response to cyclic strain. These findings support the original hypothesis that cyclic strain is a pro-inflammatory stimulus capable of modulating immune mediator expression. although these effects may occur independently of cell type.

2.5. Role of glomerular hypertension in the pathophysiology of glomerulosclerosis

Glomerular filtration and subsequent modifications of the filtrate by tubule epithelial cells are in large part responsible for fluid and electrolyte balance in the organism, thereby ensuring that the final urine volume and composition are matched to the daily intake of fluid and food. In patients with chronic renal disease and overall reductions in glomerular filtration rate (GFR), salt and water excretion is still accomplished without major dietary changes, and indeed may be achieved until such time as the GFR approaches 10% of normal. The ability of the injured/diseased kidney to adapt to these changes suggests that compensatory mechanisms occur in its remaining functioning nephrons to ensure fluid and electrolyte homeostasis. If, therefore, the initial complement of nephrons (around 2 million in humans) is damaged by disease, and diet is unchanged, then the rate of solute and water excretion per individual remaining nephrons must increase. In support of this concept, experimental studies have shown that disease processes reduce the GFR in some nephrons, whereas in less damaged nephrons compensatory changes occur to elevate the GFR and promote enlargement of tubules (reviewed in (127) and references contained therein).

2.5.1. Glomerular hyperfiltration in response to reduced nephron number

The simplest model of reduced nephron number is surgical ablation of renal tissue. In humans who have donated a kidney for renal transplantation, and in rats subjected to experimental uninephrectomy, the GFR in the remnant kidney increases by about 40%-60% above its preoperative value. Most of these changes occur in the first week following surgery. This could be potentially caused by either an increase in the

single-nephron GFR (SNGFR) of remaining nephrons, or by an increase in the actual number of nephrons in the remnant kidney. It is generally held, however, that new nephrons are not formed in mature animals, and increases in remnant kidney GFR are entirely due to increases in SNGFR. This has in fact been confirmed in the rat uninephrectomy model by micropuncture and glomerular counting techniques. Similar measurements in rats following removal of 2/3–5/6 of renal tissue shows that these adaptive increases occur similarly in superficial and juxtaglomerular nephrons.

Increases in SNGFR in both animals and humans are accompanied by increases in total renal blood flow of a similar magnitude, and this in turn represents increased perfusion of the individual glomerulus. Indeed, in the rat uninephrectomy model micropuncture techniques have demonstrated that the majority of SNGFR is due to elevation of glomerular plasma flow. Elevation of mean glomerular hydraulic pressure and alterations in the glomerular coefficient (as determined by the product of the hydraulic permeability of the glomerular capillary wall per unit surface area and the glomerular capillary surface area available for filtration) also contribute. In rats in whom greater proportions of renal mass are removed (75-90%) SNGFR of remnant nephrons was found to have increased further, and these remarkable increases were accompanied by equally prominent increases in plasma flow. This is achieved by further dilation of afferent and to a lesser extent efferent arterioles. Systemic blood pressure in this scenario, but not after uninephrectomy, is generally elevated, and accompanies an elevation in mean glomerular transcapillary pressure. This change therefore in glomerular transcapillary pressure is, therefore, important in the remarkable increases in SNGFR observed after extensive renal ablation in the rat.

The stimuli responsible for glomeruli hypertension, hyperperfusion and hyperfiltration following extensive renal ablation in the rat remain to be identified. Chronic administration of ACE inhibitors in this model has been shown to normalise the glomerular transcapillary pressure, implicating AT II in the causation of glomerular hypertension in remnant glomeruli (128). Whether the effects of AT II can occur acutely are still uncertain, and it has been speculated that AT II increases glomerular pressure in remnant glomeruli in this model by promoting Na^+ retention and promoting systemic hypertension, rather than by direct actions on the renal microcirculation (127).

Atrial naturietic peptide (ANP) contributes to elevated SNGFR in rats with reduced nephron number maintained on a liberal salt diet (129). ET-1 activity, although constant in the first week following renal ablation begins to rise thereafter (130). The significance of this is unknown. Altered prostaglandin synthesis also occurs in the remnant kidney, with increased glomerular synthesis of PGI_2, PGE_2 and TXA_2, and increased nephron excretion of the latter two (131). It has been speculated that remnant glomerular haemodynamic function may be altered by increased renal production of both vasodilator and vasoconstrictor prostanoids. In support of this, inhibition of prostaglandin synthesis both reduces remnant nephron plasma flow and SNGFR in rat renal ablation models (132) and total renal plasma flow and GFR in patients with renal insufficiency (133).

2.5.2. Compensatory renal hypertrophy in response to reduced nephron number

Both cell hypertrophy and cell hyperplasia accompany loss of renal mass and it has not been definitively determined whether hypertrophy causes hyperfiltration or vice versa. The contribution of the various structural components of the glomerulus to its expansion have been much debated (127). Overall, studies indicate that the fractions of glomerular volume occupied by its various components (capillary lumens, endothelial cells, epithelial cells and mesangium), remain constant, but it is still unclear as to what extent the different glomerular cell types increase in number and volume. Increases in proximal tubule size, and to a lesser extent distal tubule, also occur in proportion to the extent of loss of nephron number, enabling an increase in tubular reabsorption and the preservation of glomerulotubular balance. *In vitro* studies suggest, however, that in addition to increased tubule size, intrinsic adaptation of tubule transport mechanisms may also facilitate increased fluid readsorption following reduction in nephron number (127).

The observation that compensatory renal hypertrophy does not require renal innervation prompted the search for circulating agents or hormones which could control renal growth. No renal-specific factor has been identified to date, but IGF-1, EGF, PDGF, PGE_2, in addition to the hormones thyroxine, hydrocortisone, vasopressin and AT II have all been shown to promote growth of renal cells in culture, in particular mesangial and proximal tubule cells (127). It has been speculated that these factors may participate, in a non-specific way in renal hypertrophy, after growth has been triggered by some, as yet unidentified, signal.

2.5.3. Function of intact nephrons in parenchymal renal disease

Compensatory elevation of SNGFR must be accompanied by increased proximal water and solute reabsorption to avoid inappropriate diuresis and circulatory collapse. The converse is also true with a reduction in SNGFR (134). Micropuncture studies in various models of experimental glomerulonephritis and tubulointerstitial damage have confirmed maintenance of this so-called glomerulotubular balance over a wide range of SNGFR values (135). In the case of experimental GN, alteration in Starling forces governing peritubular capillary reabsorption have been shown to account for the appropriate coupling of proximal Na^+ and water retention to glomerular filtration. Oncotic pressure in the efferent arteriole was found to vary directly with SNGFR, while the hydraulic pressure was similar throughout the peritubular capillary network. Structural changes then in the proximal tubule and peritubular capillary network appear to act in concert with changes in Starling forces to achieve glomerulotubular balance.

Studies in human renal disease are generally in agreement with the above concepts, although information is less complete. It has been demonstrated, for example, that hypertrophied tubules are most often attached to hypertrophied glomeruli and shrunken tubules to shrunken glomeruli.

2.5.4. Adverse effects of glomerular capillary hypertension: glomerulosclerosis

Patients with established renal insufficiency generally display progressive loss of renal function. Those in whom the GFR has been reduced to about 25% of normal can expect to eventually require dialysis or transplantation (127). It appears then, that

after a certain point, reduction in functioning nephron numbers, irrespective of the cause, leads to progressive failure of remaining units through a final common pathway of injury. Mounting evidence suggests that the haemodynamic changes causing remnant glomerular hypertension and hyperfiltration, although initially adaptive, may eventually cause injury to residual glomeruli. In the rat renal ablation models, early glomerular hypertension, hyperfiltration and hypertrophy is accompanied by ultrastructural changes in epithelial cells including fusion of their foot processes (136). Following this focal detachment of endothelial and epithelial cells from the GBM occurs. Finally, progressive accumulation of subendothelial hyaline material and collapse of capillary lumens eventually results in the appearance of focal and segmental glomerulosclerosis. The numbers of sclerotic lesions increases with time and progression to global sclerosis is observed while the pace of the injury, like the degree of increase in SNGFR, increases in proportion to the loss of renal mass (136).

Restriction of dietary protein lowers GFR in normal animals. Studies investigated whether this dietary regimen could effect a lowering of the glomerular pressure and flow in rats following extensive renal ablation. Restriction of dietary protein was found to reduce proteinuria and early glomerular morphological changes as compared to rats following ablation fed on standard chow (137). This paradigm also holds in models of less extensive renal ablation where protein restriction results not only in functional improvements, but a longer life-span. Late institution of protein restriction also limits the progression of established renal injury and studies indicate that this is via a reduction in glomerular capillary pressure, without a reduction in remnant glomerular hyperfiltration and hyperperfusion (138). In support of the role

of increases in glomerular capillary pressure in the development of glomerulosclerosis, in a rat remnant kidney model in which animals develop glomerular hyperfiltration without glomerular hypertension, glomerular injury is absent (139).

Glomerulosclerosis is a characteristic of animal models of experimental hypertension where increases in systemic pressure are transmitted to the glomerulus and cause capillary hypertension. Blockade of nitric oxide synthesis, or sustained infusion of AT II, cause systemic and glomerular hypertension and the rapid development of glomerulosclerosis in intact rats with normal nephron number (128, 140). In the two-kidney one-clip model of hypertension, rats develop glomerular hypertension and glomerulosclerosis only in the unclipped kidney (141). In contrast, in models where the glomerular pressures remain normal despite elevated systemic pressures (SHR, Milan hypertensive rats) early glomerulosclerosis is not prominent (142). Use of ACE inhibitors in rats subjected to renal ablation has been shown to control systemic blood pressure, normalise glomerular capillary pressure and prevent glomerular injury. Interestingly, ACE inhibitors have been shown to provide some protection against glomerular injury in every model of renal disease in which they have been tested to date.

A number of other non-haemodynamic mechanisms may also contribute to remnant nephron injury (127). The finding of lipid droplets in glomerulosclerotic lesions led to the hypothesis that glomerular deposition of circulating lipids could contribute to progressive glomerular injury in renal disease (143). Lipid feeding in rabbits and guinea-pigs causes glomerular inury. In the rat renal ablation model and in rats with

hereditary obesity, hyperlipidaemia is also associated with progressive glomerular injury and, indeed, lipid lowering in these models limits glomerulosclerosis (127). The lipoproteins causing injury are as yet unidentified and indeed the observation that cholesterol feeding itself can increase glomerular pressure causes some difficulty in the interpretation of these findings.

Capillary microthrombi also occur after reduction in nephron number (127). It has been speculated that these are precipitated by early endothelial cell injury in the remnant glomeruli and contribute to progressive injury and glomerulosclerosis by occlusion of capillary lumens and release of platelet-derived factors. This prompted studies of anti-coagulant drugs in the rat renal ablation model and, while heparin largely prevents glomerulosclerosis, warfarin and aspirin are less effective. Heparin may have other effects in this model such as a reduction in blood pressure, so the role of capillary thrombosis in this glomerulosclerosis requires further clarification.

2.5.5. Glomerular hypertension: cellular mechanisms of glomerular injury

The different mechanisms suggested for glomerular injury are not mutually exclusive and it is probable that each contributes, to a greater or lesser degree, to the development of glomerulosclerosis. Initial theories suggested that increases in glomerular pressure resulted in the movement of macromolecules into the mesangium. This was subsequently demonstrated in the rat renal ablation model where proteinuria is due to defects in the charge and size selective properties in the capillary wall (as measured by dextran sieving studies) (144). Once present in the mesangium, it has been speculated that these macromolecules may stimulate

mesangial cell proliferation, recruit mononuclear cells into the mesangium and alter production of extra-cellular matrix components.

Movement of proteins through the glomerular capillary wall may also cause tubulointerstitial damage. Many progressive glomerulopathies have in common persistently high levels of urinary protein excretion and tubulointerstitial lesions at biopsy (145, 146). The functional importance of tubulointerstitial events in progressive renal failure is supported by evidence that that the severity of tubulointerstitial damage and in particular macrophage infiltration correlates strongly with the risk of progressive renal dysfunction, even more so than glomerular lesions (145, 146). Because more severe proteinuria is associated with more severe lesions and faster progression of renal damage, and high protein loads on the kidney experimentally causes structural parenchymal injury and interstitial macrophage accumulation, it has been speculated that proteins filtered through the glomerular capillary may have intrinsic renal toxicity (so-called "protein overload hypothesis"). In support of this concept, studies have shown that in remnant kidneys following 5/6 renal mass ablation, albumen and IgG accumulation by proximal tubular cells was apparent at early time points and preceded interstitial infiltration of MHC-II-positive cells and macrophages (147). Using double-staining techniques, infiltrates were visualised at or near tubules containing intracellular IgG or luminal casts. Further studies also showed the colocalisation of complement (C3) and the chemokine osteopontin to these regions. Interestingly, similar patterns were found in an immune model (passive Heymann nephritis) (147), suggesting that the interstitial inflammatory reaction develops at sites of protein overload, regardless of the type of

glomerular injury. The mechanisms underlying the proinflammatory response of tubular cells to protein challenge have yet to be established.

Other processes of injury in response to glomerular hypertension are shown in the figure below (Fig 2.16).

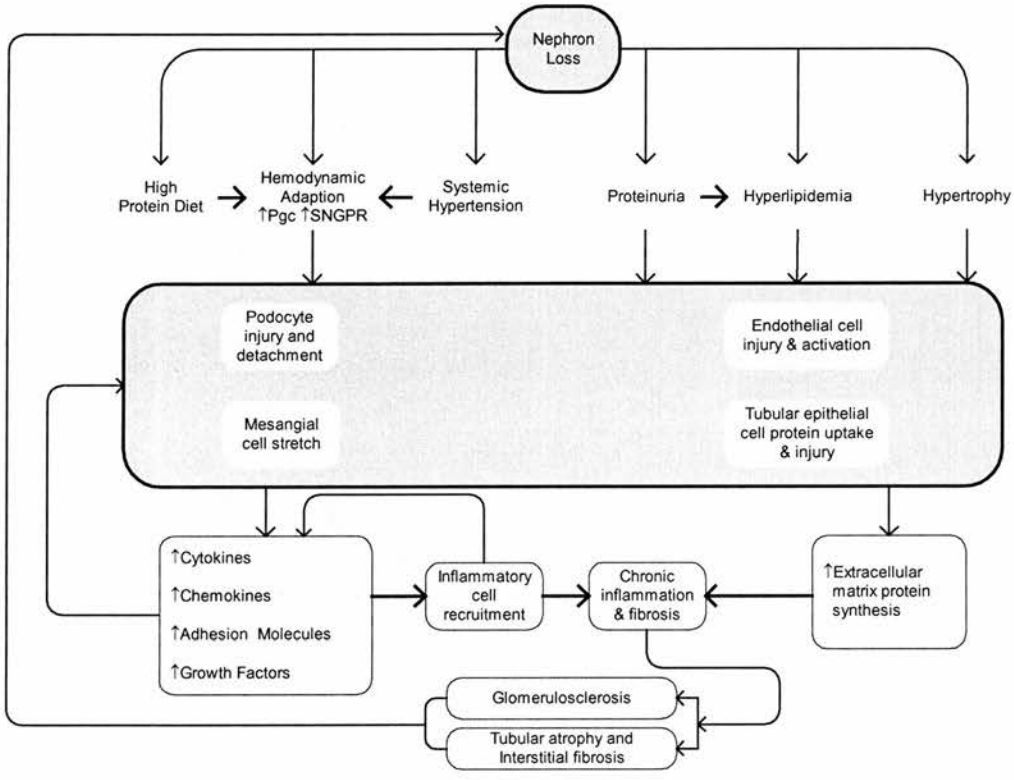


Figure 2.16.Potential mechanisms of glomerular injury in the development of glomerulosclerosis (adapted from (127)).

Serial studies in rats subjected to renal ablation suggest that all these processes occur in parallel and at this time it is impossible to identify any one particular initiating or predominant feature in the development of glomerulosclerosis.

In a recent study screening for genes upregulated in the 5/6 nephrectomised mouse kidney using a PCR-based subtraction method, 10 known and 9 novel genes were identified. 12 clones showed a 1.5- to 6-fold upregulation by northern blot analyses, while the remaining seven were rarely expressed (148). The known genes included kidney androgen-related protein, major urinary protein, lysozyme M, metalloproteinase-3 tissue inhibitor, chaperonin 10, cytochrome oxidase I, epsilon-sarcoglycan, ribosomal protein S3a, G-proteingamma10 subunit and splicing factor 9G8. These suggest a possible role for androgen action, mitochondrial functions, matrix metabolism, cell-matrix interactions and intracellular signalling events in the initial hyperfiltration and hypertrophy of remnant kidney.

2.5.6. Putative link between glomerular hypertension and immune cell infiltration

It has been speculated that immune cell infiltration of the hypertensive glomerulus may promote glomerular injury. Certainly in animal models, leukocytes accumulate in the mesangium of injured remnant glomeruli (149). Interestingly, depletion of leukocytes in rats by irradiation delays the onset of glomerular injury after renal ablative surgery while mycopenolate mofetil, an immunosuppressive agent selective for T lymphocytes, results in partial remission of renal injury in this model. (149, 150). In human diabetic glomerulosclerosis (which is preceded by glomerular hyperfiltration), macrophage (but not T or B cell) infiltration appears to occur in the stage of moderate diabetic glomerulosclerosis (as opposed to the mild or advanced stages) and it has been suggested that this transient macrophage infiltration may contribute to irreversible structural damage by the augmentation of ECM production or the production of inflammatory mediators (151). Potential cytokines participating

in glomerular injury include all those made by the native glomerular cells in addition to those released by infiltrating immune cells.

Evidence has been presented which supports the upregulation of cellular adhesion molecules within the remnant glomeruli as a potential mechanism for mononuclear cell recruitment. Levels of glomerular ICAM-1 expression are significantly increased in the 5/6 nephrectomised rat model as compared to sham operated controls and correlate with mononuclear leukocyte infiltration. Anti-ICAM-1 monoclonal antibody effectively prevents this infiltration of macrophages into the glomerulus following nephrectomy (152). Treatment of streptozotocin-induced diabetic rats with an aldose reductase inhibitor, which reduces glomerular hyperfiltration but does not affect glucose levels, reduces ICAM-1 protein expression and mononuclear cell infiltration (153). Moreover, mononuclear cell infiltration into the diabetic glomerulus has also been shown to be prevented by anti-ICAM monoclonal antibody, and both ICAM-1 protein expression and cellular infiltration were decreased by insulin treatment. These results suggest that glomerular hypertension and hyperfiltration may promote glomerular macrophage infiltration by the induction of ICAM-1 expression.

2.5.7. Glomerular pressure and cyclic mechanical strain

Under normal conditions, because glomeruli are exposed to only small pulse pressure variations and not to the low frequency oscillations in systemic pressure, volume remains stable. The contractile activity of the afferent arteriole provides effective autoregulation and tight control of intra-glomerular pressure. The pre-glomerular dilatation that characterises a wide range of renal diseases, however, facilitates the

delivery of a greater fraction of the systemic pressure, normal or elevated, to glomerular capillaries. In many models of progressive renal disease such as the remnant and diabetic kidney, autoregulatory glomerular protection is impaired and marked augmentation of the moment to moment variations in pressure occurs within the glomerulus (154). This, in turn, results in wide swings in glomerular volume, which can be further exacerbated by the systemic hypertension that often co-exists with renal disease. In normal glomeruli with intact autoregulation moment to moment volume varies by a mere 0.4%, while volume changes of up to 7.4% can occur in glomeruli from remnant kidneys of hypertensive animals (154). These changes in overall volume are associated with parallel changes in all the glomerular structural components. In the case of a mesangial cell, which is anchored via cytoplasmic projections to the peripheral basement membrane, pressure induced glomerular expansion and distended capillaries will displace these anchoring points and produce intense mesangial cell stretch. It has been suggested, therefore, that cyclic changes in glomerular volume result in repeated episodes of mesangial stretch and relaxation.

The degree to which elevated intraglomerular pressure will deform or stretch an endothelial or mesangial cell depends on the mechanical compliance of the glomerulus and supporting structures; the greater the compliance, the greater the stretch. Studies on the glomeruli of subtotally nephrectomised rats have shown changes in their elastic properties that result in increased mechanical compliance (154). As a consequence, any increase in intraglomerular pressure will tend to subject mesangial cells to a greater degree of deformity or mechanical strain. In addition, at any given glomerular pressure, larger, hypertrophied glomeruli are more

distensible than smaller glomeruli, most likely secondary to increased capillary wall tension in hypertrophied capillaries. Consequently, glomerular hypertrophy, whether induced by nephron loss or diabetes, magnifies the deleterious effects of altered glomerular haemodynamics (69).

ECM accumulation is the principle pathognomic hallmark of glomerulosclerosis (127). The expanded mesangial matrix contains normal matrix components including laminin and type IV collagen as well as atypical components such as types I and III collagen. Matrix accumulation results both from increased production and decreased degradation of ECM components. Matrix breakdown is the function of the matrix metalloproteinases (MMPs). The activity of the MMPs is inhibited by the Tissue Inhibitors of Metalloproteinases (TIMPs), a group of endogenous regulatory proteins. Increased production of ECM components, diminished metalloproteinase activity or increased TIMP activity may contribute to, and/or, individually cause ECM accumulation.

How increased biomechanical forces, in particular cyclic stretch-relaxation, are translated into extracellular matrix accumulation and glomerulosclerosis has been the focus of recent investigations. Working with glomeruli isolated from rat kidneys, Riser and co-workers undertook a series of microperfusion experiments (155). Changes in volume in microdissected rat glomeruli perfused *ex vivo* at increasing flows, were recorded and plotted against the corresponding intraglomerular pressure. At perfusion pressures known to occur pathologically *in vivo*, a 16% increase in glomerular volume was observed. Using this information, Riser subjected cultured rat mesangial cells to cycles of 16% stretch using the Flexercell unit (described in

detail in section 2.3.1) (155). The strained mesangial cells increased their synthesis of types I and IV collagen, laminin and fibronectin, suggesting that corresponding pathological mechanical strain *in vivo* may produce a similar response. Interestingly, the accumulation of ECM induced by cell stretch is markedly enhanced when the ambient glucose concentration is increased.

2.5.8. Mesangial cell mediators released in response to mechanical strain

Evidence is mounting, of a pre-eminent role TGF-β in the accumulation of ECM in glomerulosclerosis. Isaka and co-workers have shown that transfection of TGF-β1 results in expansion of the mesangial matrix in an *in vivo* rat model (156), while inhibition of TGF-β has been shown to protect against scarring in experimental models of renal disease (157). TGF-β has been specifically implicated in the development of DN (158), where production by mesangial cells has shown to be a consequence of both mechanical (159) and biochemical stimuli (160). Participation of TGF-β in the mesangial cell response to mechanical strain has been demonstrated by several investigators. Increases in TGF-β1 mRNA can be detected following 24 hours of cyclical strain of rat MCs, while increases in TGF-β1 protein can be demonstrated by 48 hours (159). In the same set of experiments, Northern blot analysis showed increases in mRNA for matrix proteins types I, III and IV collagen, laminin and fibronectin to be maximal after 12 hours of cyclical strain suggesting that other factors in addition to TGF-β are involved in the early regulation of matrix protein accumulation. Northern blot analysis also revealed a significant decrease in mRNA for matrix metalloproteinase-2 (MMP-2) within 6 hours of initiation of stretch/relaxation. In contrast, expression of tissue inhibitor of metalloproteinase-2

(TIMP-2) was found to be increased within 12 hours of stretch/relaxation. The net effect of these two changes would be a significant reduction in the rate of matrix breakdown, facilitating overall matrix accumulation. It seems likely that net matrix accumulation occurs as a result of a shift in the balance between these opposing trophic and degradative factors.

Some clues as to the mechanism of action of TGF-β were provided by Hori et al (161). TGF-β is secreted as a large latent complex (LLTC) associated with latent TGF-β binding protein-1 (LTBP-1), the latter molecule being known to bind ECM. LTBP-1 mRNA expression in rat MCs has been shown to be elevated during the first 24 hours of stretch and then decreased over the next 12 hours. Moreover anti-LTBP-1 antibodies inhibited fibronectin and laminin mRNA expression normally induced by stretch-induced TGF-β. Of note the antibodies did not prevent TGF-β interacting with its receptor nor did they diminish its production by the MCs suggesting the target of inhibition may be at the stage of TGF-β activation through inhibition of the LTBP-ECM association.

2.5.9. Mechanical strain to functional response: putative signal transduction mechanisms

Two alternative theories attempt to explain the process of transduction of mechanical strain to functional response in the stretched mesangial cell. These are discussed in detail in an earlier section (2.3.3) focusing on endothelial cells and cyclic strain, but the underlying concepts are broadly similar. The first paradigm relates to the observation that stretch can activate mechanosensitive ion channels in the cell membrane. Stimulation of ion fluxes (potassium, calcium and possibly other) across

these channels could also potentially activate signal transduction cascades. The second theory proposes that mechanical strain sensed by cell surface adhesion plaques and cytoskeletal proteins, leads to second-messenger mobilisation.

Specific stretch-activated (SA) cation channels have been described in mesangial cells. Akai and co-workers recorded the presence of stretch-induced calcium fluxes in mesangial cells and linked Ca^{2+} influx to the induction of the protooncogenes *c-fos* and *egr-1* via protein kinase C activation (123). Moreover, the combination of nifedipine (a calcium channel blocker) and gadolinium chloride (a specific SA channel blocker) has recently been shown to prevent stretch mediated upregulation of *c-fos,* indicating L-type voltage-gated calcium channels may also be involved in stretch-induced calcium mobilisation. These results have also led to speculation that ion channel blockers may hold promise as therapies for ameliorating hypertension induced glomerular damage (162).

Working with rat mesangial cells, Craelius et al. assessed the potential role of mechanically-activated potassium and sodium ion channels (MACs) as signal transducers in the alteration of mesangial cell morphology known to occur in response to mechanical stretch (163). MACs have been identified on numerous cell types and have been implicated in the electromechanical coupling which mediates a depolarisation which in turn triggers stretch-induced contraction (164). Mesangial cells are richly endowed with MACs and these channels have been suggested to play a role in mesangial cell response to hyperosmolar states. Craelius et al. demonstrated that cell swelling or stretching of small portions of the cell membrane such as would occur in glomerular hypertension led to MC MAC activation. The channels carry a

large current composed of potassium and sodium. The authors proposed this could confer mesangial cells with both osmosensitive and mechanosensitive properties, allowing them to respond to intraglomerular physical forces. In support of the role of ion channels in mediating glomerular hypertension-induced pathology, ion channel regulation has recently been linked causally to hyperfiltration during the early stages of diabetes mellitus (165). However, the exact role of ion channels in the development of glomerulosclerosis remains to be elucidated.

Mechanical forces will exert their greatest effect at the points of attachment of the cell to the underlying ECM, - focal adhesion plaques. Rapid tyrosine phosphorylation of FAK and paxillin, protein components of the focal adhesion plaques, has recently been reported for stretch-stimulated rat mesangial cells (166). Clustering of β1-integrin toward the periphery of the mesangial cell and stress fibre formation was demonstrated in the same study. While inhibition of integrin-ECM binding failed to suppress this process, inhibition of PKC and PLC and use of the intracellular calcium chelator BAPTA-AM completely blocked it. Activation of PKC and PLC and intracellular calcium mobilisation (possibly via stretch-activated ion channels) seem to be required to facilitate the focal adhesion and cytoskeletal response to mechanical strain. It would appear therefore, the ion flux and cytoskeletal based paradigms may not be mutually exclusive alternatives. The correct paradigm is likely to contain elements of both.

2.5.10. Regulation of transcription factor activation and gene expression by mechanical forces in mesangial cells

Cyclic strain has been demonstrated to activate protein kinase C, increase intracellular calcium concentrations and induce tyrosine kinase phosphorylation of focal adhesion plaque proteins in mesangial cells. Stimulation of resting mesangial cells by serum or vasoconstrictors results in a rapid and transient expression of the protooncogenes *c-fos* and *zif 268/egr-1* (167). Akai et al have demonstrated stretch/relaxation induced-expression of these protooncogenes, as well as the mitogen-inducible cyclooxygenase (COX-2), in cultured rat mesangial cells (123). The induction of these immediate early genes was preceded by activation of protein kinase C (PKC) and calcium influx. By 24 hours of cyclical stretch/relaxation, PKC activity had returned to basal levels and its distribution was similar to that in unstimulated cells. Inhibition of PKC had a profound inhibitory effect on subsequent stretch/relaxation induced gene expression. Stimulating the mesangial cells in medium deficient in calcium also diminished protooncogene production. Taken together the results suggest an important role for both PKC and calcium dependent mechanisms in mesangial cell responses to mechanical strain. In addition to protein kinase C activation and calcium influx, stimuli that lead to *c-fos* induction, also require activation of mitogen activated protein (MAP) kinase pathways. Of the three MAP kinase pathways described in mammalian cells, work from Ingram et al. has revealed only *ERK 1/2* and *p38* MAPK are activated in stretched mesangial cells (168).

2.6. Influence of cyclic mechanical strain on glomerular endothelial and mesangial cell activation *in vitro*

2.6.1. Introduction

How increased biomechanical forces occuring in glomerular hypertension, in particular cyclic stretch-relaxation, are translated into glomerular injury, and, ultimately, glomerulosclerosis have not been fully elucidated. Increased intraglomerular pressure promotes glomerular expansion and, it has become widely accepted, compliant stretching of resident glomerular cells. In addition, the pre-glomerular dilatation that characterises a wide range of renal diseases facilitates the delivery of a greater fraction of the systemic pressure, normal or elevated, to glomerular capillaries. In many models of progressive renal disease such as the remnant and diabetic kidney, autoregulatory glomerular protection is impaired and marked augmentation of the moment to moment variations in pressure occurs within the glomerulus (69). This in turn results in wide swings in glomerular volume, which can be further exacerbated by the systemic hypertension that often co-exists with renal disease. *In vitro* studies have provided evidence for the involvement of increased cyclic mesangial cell stretch in the sclerotic processes of glomerular injury (155, 159). Evidence has also been presented which suggests that leukocyte infiltration of the glomerulus, an event critical to the pathogenesis of inflammatory-based glomerular diseases, may play a role in the development of glomerulosclerosis (149, 151). The importance of mechanical stretch, however, as a putative pro-inflammatory stimulus capable of the modulation of immune mediator expression and leukocyte trafficking determinants within the glomerulus, has not yet been

examined. The aim of this study was to examine the influence of cyclic mechanical strain on glomerular endothelial and mesangial cell activation *in vitro*.

2.6.2. Materials and Methods

Cell Culture

All cell culture reagents were obtained from Gibco Life Technologies (Paisley, Scotland) unless otherwise stated. SV40 transformed human mesangial cells (tHMC) (169) were a kind gift from Dr. Ann McGinty. These cells were routinely cultured in RPMI 1640 (BioWhitaker, Maryland, USA), supplemented with 5% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin.

Transformed rat glomerular endothelial cells (RGEC) were a generous gift from Dr. Harry Holthofer and have been characterised previously (170). These cells were routinely cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing L-alanyl-L-glutamine, sodium pyruvate, 1000 mg/L glucose and pyridoxine and supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin.

Application of Cyclic Mechanical Stretch to tHMC and RGEC

tHMC ($6x10^5$ cells / well) were seeded on elastin-coated, flexible and rigid based six-well plates, (Flex I and II plates respectively, FlexerCell, McKeesport, PA, USA). Growth media was replaced with RPMI, containing 0.5% FCS 1 Hr prior to application of cyclical mechanical stretch.

RGEC were seeded on to on collagen-coated, flexible and rigid based six-well plates, (Flex I and II plates respectively, FlexerCell, McKeesport, PA, USA). Prior to experiments RGEC were not rendered quiescent, since they were found to detach under conditions of serum restriction.

Stretch was induced using the Flexercell Strain Unit (FX-2000). This instrument, described in detail in section 2.3.1, provides precisely timed, negative pressure cycles of controlled magnitude, deforming the base of the culture plate and stretching the cells growing on the upper surface of the well. Previous work from Riser et al has shown microdissected rat glomerular volume to increase by 17% over a pathophysiological intraglomerular pressure range likely to occur *in vivo* in the setting of glomerular hypertension (155). Thus to mimic conditions of cyclical pulsatile strain present in the glomerulus in hyperfiltration states, we exposed the cultured tHMC and RGEC to continuous cycles of stretch/relaxation at a rate of 60 cycles per minute (using alternate cycles of 0.5 seconds stretch followed by 0.5 seconds of relaxation) at a vacuum pressure of –15 kPa to induce maximal elongation of 17%.

Northern Blot Analysis and RT-PCR

Total cellular RNA was isolated using Trizol reagent according to the manufacturer's instructions. Total RNA (5μg) from tHMC was subjected to Northern blot analysis for fibronectin expression exactly as described previously (171). Product quantification was achieved by normalisation to GAPDH, by means of phosphorimaging. Total RNA (2μg) (tHMC and RGEC) was treated with DNase I and Oligo$(dt)_{12\text{-}18}$ was then used to generate first strand cDNA. Reverse transcription was carried out using Superscript II

RNase H. Sequence specific primer pairs were either obtained from Biosource (Camarillo CA), or designed and obtained from Sigma-Genoys UK (Tables 2.4, 2.5).

Human Gene	Annealing Temp. °C	Product Length, bp	Sequence P1= Primer 1 P2= Primer 2
Chemokines			
MCP-1	60	279	Obtained from BioSource Int. CA, USA
IL-8	60	227	
RANTES	60	197	
Cytokines			
TGF-β	55	186	**P1** 5'-CAG AAA TAC AGC AAC AAT TCC TGG 3' **P2** 5'-TTG CAG TGT GTT ATC CGT GCT GTC 3'
CTGF	58	348	**P1** 5'-GGT TAC CAA TGA CAA CGC CT-3' **P2** 5'- AAG ATG TCA TTG TCT CCG GG-3'
IL-1β	60	331	**P1** 5'-CTT CAT CTT TGA AGA AGA ACC TAT-3' **P2** 5'-AAT TTT TGG GAT CTA CAC TCT CCA-3'
TNF-α	60	325	**P1** 5'-CAG AGG GAA GAG TTC CCC AG-3' **P2** 5'-CCT TGG TCT GGT AGG AGA CG-3'
IFN-γ	60	510	**P1** 5'-ATG AAA TAT ACA AGT TAT ATC TTG GCT TT-3' **P2** 5'-GAT GCT CTT CGA CCT CGA AAC AGC AT-3'
IL-6	60	501	Obtained from BioSource Int. CA, USA
Chemokine Receptors[1]			
CCR1	56	327	**P1** 5'-ACC TGC AGC CTT CAC TTT CCT CAC-3' **P2** 5'-GGC GAT CAC CTC CGT CAC TTG-3'
CCR2	56	255	**P1** 5'-CCA ACT CCT GCC TCC GCT CTA-3' **P2** 5'-CCG CCA AAA TAA CCG ATG TGA TAC-3'
CXCR2	57	385	**P1** 5'-CCG GGC GTG GTG GTG AG-3' **P2** 5'-TCT GCC TTT TGG GTC TTG TGA ATA-3'
GAPDH			
GAPDH	60	495	**P1** 5'- ACC ACA GTC CAT GCC ATC AC-3' **P2** 5'- TCC ACC ACC CTG TTG CTG TA-3'

1. Reference (100)

Table 2.4. Human primer sequences and product sizes.

Amplification was as previously described, typically 94^0C for 3 minutes; 35-40 cycles of 94^0C for 30 seconds (denaturing), 56^0C-60^0C for 1 minute (annealing) and 72^0C for 1 minute (extension); followed by a final extension step of 72^0C for 7 minutes. cDNA samples were subjected to parallel PCR reactions with primers for GAPDH to control for equivalency of loading. The presence of genomic DNA was determined by control reactions in which amplification was conducted in complete reaction mixture lacking template cDNA or with RNA samples from RT reactions carried out in the absence of Superscript II. PCR products were visualised by ethidium bromide staining following electrophoresis on 1.2% agarose gels. Subsequent quantification for each gene studied

in comparison to the GAPDH loading control was conducted using the GelWorks 1D software package (UVP, Cambridge UK).

Rat Gene	Annealing Temp. °C	Product Length, bp	Sequence P1= Primer 1 P2= Primer 2
Adhesion Molecules			
ICAM-1	60	600	**P1** 5'-TTC TGC CAC CAT CAC TGT GT-3' **P2** 5'-TTC TCC ATC TCC AGG GTC TG-3'
VCAM-1	60	451	**P1** 5'-GAC CTG TCA GCG AAG GAA AC-3' **P2** 5'-TGA GCA GGT CAG GTT CAC AG-3'
Chemokines			
GRO-α	60	180	**P1** 5'-AGA CAG TGG CAG GGA TTC AC-3' **P2** 5'-ACT TGG GGA CAC CCT TTA GC-3'
Cytokines			
TGFβ	60	552	**P1** 5'- GTC AAC TGT GGA GCA ACA CG-3' **P2** 5'-TGG TTG TAG AGG GCA AGG AC -3'
Chemokine Receptors			
CCR1	60	503	**P1** 5'- AAA CCT ACC CCA CAA CCA CA-3' **P2** 5'-CTG GGC CTT GAA AAA GCA TA -3'
CCR2	60	350	**P1** 5'-TTT GAT CCT GCC CCT ACT TG-3' **P2** 5'-TTT TGG CAA TGT GCT TTC TG-3'
CXCR2	60	197	**P1** 5'-AGT TCT GAC CCG CCC TTT AC-3' **P2** 5'-GCC AGG TTC AGC AGG TAG AC-3'
GAPDH			
GAPDH	60	495	**P1** 5'- ACC ACA GTC CAT GCC ATC AC-3' **P2** 5'- TCC ACC ACC CTG TTG CTG TA-3'

Table 2.5 Rat primer sequences and product sizes.

2.6.3. Results and discussion

In order to validate the conditions chosen for cyclic mechanical stretch in the current study, tHMC were subjected to a maximum elongation of 17% using alternate 0.5 s cycles of stretch and relaxation for 24, 48 and 72 Hr. Total cellular RNA was then isolated and subjected to Northern blot analysis using a cDNA probe for fibronectin, a gene previously shown to be upregulated in mesangial cells in response to this stimulus (159). As can be seen (Fig. 2.17), under these conditions of cyclic mechanical stretch fibronectin expression was increased in tHMC at 24, 48 and 72 Hr (1.78, 1.82 and 1.61 fold over control, respectively).

Mesangial cell expression of the cytokine TGF-β has also been shown increase in response to cyclic strain. In the current study, therefore, expression of this cytokine in tHMC following exposure to cyclic strain was examined. For this and subsequent studies, total cellular RNA was isolated and subjected to RT-PCR. mRNA levels for this species were increased by 48 and 72 hours exposure to cyclic mechanical stretch (2.0 and 2.8 fold/basal respectively) (Fig 2.18). The finding that two stretch responsive genes, fibronectin and TGF-β, were upregulated in tHMC in response to cyclic mechanical stretch validated the experimental parameters chosen for the current study.

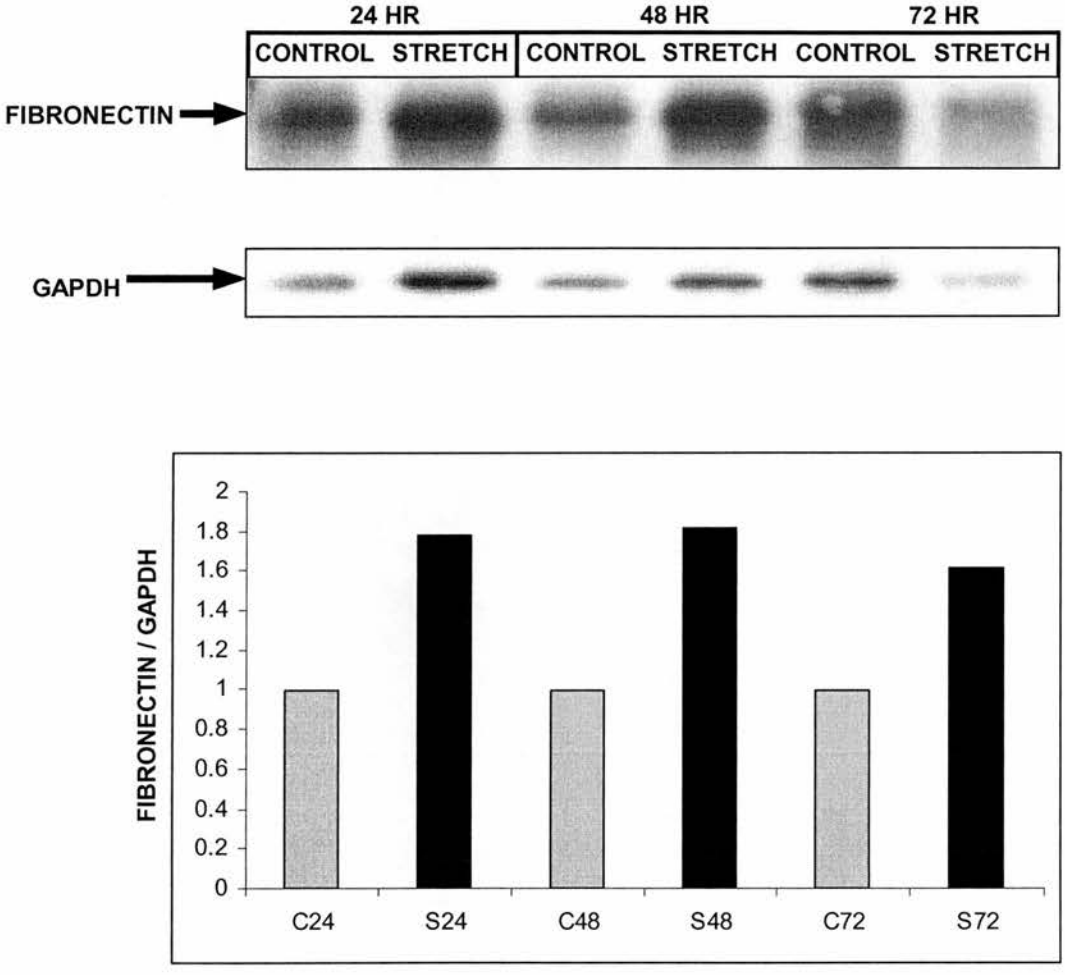


Figure 2.17 Fibronectin expression is increased in tHMC cells in response to cyclic strain.

Expression of fibronectin under basal conditions is apparent, with increases in mRNA for this species occuring following exposure to 24, 48 and 72 hours of mechanical strain. Result shown is representative of three separate experiments.

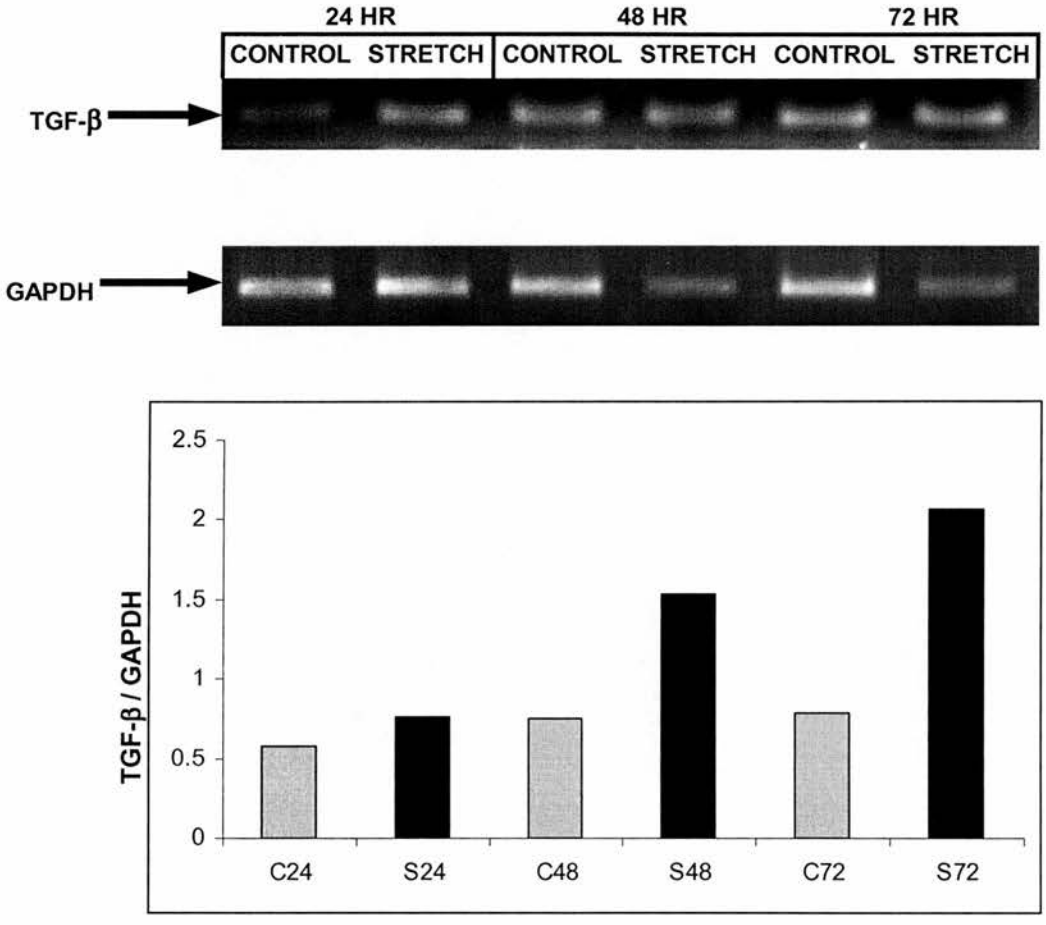


Figure 2.18 TGF-β expression is increased in tHMC in response to cyclic strain

Basal expression of TGF-β is apparent in tHMC with increases in mRNA for this cytokine occuring in response to 48 and 72 hours of cyclic strain. Result shown is representative of three separate experiments (Appendix 1B).

ECM accumulation is the principle pathognomic hallmark of glomerulosclerosis. The findings presented above are consistent with the hypothesis that glomerular hypertension, via increases in glomerular expansion and mesangial stretch, is a profibrotic stimulus. The induction of TGF-β and its subsequent actions in an autocrine/paracrine manner enhance ECM synthesis and deposition by mesangial cells and, in agreement with these observations, increases in TGF-β expression have been demonstrated in various forms of proliferative glomerulonephritis. The complex actions of this cytokine are discussed in detail in section 3.5.1 but it has been suggested that in the context of glomerulosclerosis the pro-fibrogenic effects of this cytokine, rather than its anti-inflammatory properties, predominate.

Expression of the cytokine connective tissue growth factor (CTGF) is potently induced by TGF-β and is a putative downstream mediator of TGF-β action on mesangial cells and fibroblasts, where it stimulates synthesis of ECM via increases in collagen type 1 and fibronectin production (172). Our group has recently demonstrated that high glucose stimulates mesangial CTGF expression *in vitro* by TGF-β- and protein kinase C-dependent pathways (173). Moreover, glomerular CTGF expression is elevated in steptozotocin-induced diabetes (174). On this basis, it was speculated that CTGF may be a mediator of TGF-β-driven matrix production in diabetic glomerulosclerosis The demonstration that CTGF mRNA is upregulated in several human glomerulopathies suggests that CTGF may also be an important stimulus for glomerulosclerosis in other forms of progressive renal disease (175). Induction of CTGF (mRNA and protein) in rat glomeruli has been demonstrated at 8 and 12 weeks post-renal ablation (a model of glomerular hyperfiltration), while

studies *in vitro* have demonstrated increases in mesangial CTGF expression and secretion in response to the cytokines IL-1β, IL-4, TNF-α and TGF-β (176).

Given the evidence supporting a role for CTGF in the pathogenesis of glomerulosclerosis, the influence of cyclic mechanical strain on expression of this species in tHMC was examined. Although the expression of this mediator was not increased following 24 hours of cyclic strain, dramatic elevations in mRNA do occur in response to 48 and 72 hours of this stimulus (Fig 2.19). Strain-induced CTGF expression in primary human mesangial cells has recently been demonstrated in our laboratory (Gupta et al., unpublished observations) and by other investigators (177). The increases in CTGF expression at 48 and 72 hours occur in tandem with strain-induced increases in TGF-β expression; work is ongoing to establish whether these effects are mediated by TGF-β. These results provide evidence that increased biomechanical forces occuring in the setting of glomerular hypertension could potentially, via increases in CTGF, result in the ECM accumulation characteristic of glomerulosclerosis.

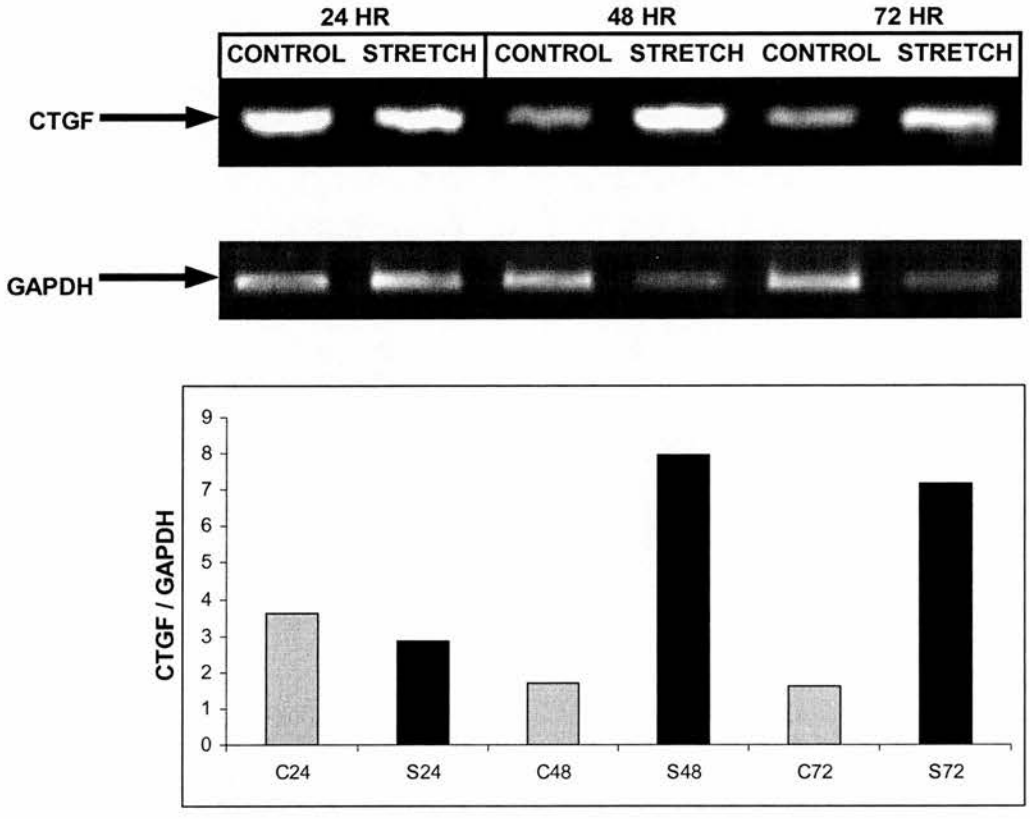


Figure 2.19 CTGF expression in tHMC is increased in tHMC following cyclic strain.

Basal expression of CTGF is apparent with increases in mRNA levels for this species occuring in response to 48 and 72 hours of cyclic strain. Result shown is representative of three separate experiments (Appendix 1B).

The glomerular and interstitial infiltration of leukocytes is a common phenomenon found in most renal diseases, independent of whether they are immune or non-immune in origin. In the scenario of glomerular hypertension, leukocyte infiltration, predominantly macrophages, has been demonstrated in experimental models of hyperfiltration and in certain human renal diseases which progress to glomerulosclerosis (section 2.5). The role of these cells, however, in the development of human glomerulosclerosis is still unknown. Conditions that determine the cellular infiltrate at the site of injury include the local production and release of chemokines in addition to the activation of both infiltrating inflammatory cells and resident renal cells. A role for chemokines in renal diseases has been suggested in experimental animal models where chemokine mRNA is increased in timely concordance with macrophage infiltration (e.g, elevated MCP-1 in anti-Thy 1 nephritis and nephrotoxic nephritis) (178, 179). Furthermore, in human kidney disease, the expression of various chemokines in biopsy specimens at both mRNA and protein level (MCP-1, RANTES, IL-8) correlates with immune cell infiltration (180).

It is apparent that mesangial cells, as discussed in section 2.5, rather than being a passive target for the action of infiltrating cells, are themselves important participants in glomerular injury. Indeed studies *in vitro* have demonstrated the upregulation of mesangial cell adhesion molecules and their elaboration of cytokines/chemokines in response to various pro-inflammatory stimuli. On the basis that cyclic strain is a putative pro-inflammatory stimulus, the expression of the chemokines IL-8, RANTES and MCP-1 in tHMC in response to this stimulus was examined.

Expression of IL-8 is reduced following 24 and 48 hours of cyclic mechanical stretch (with levels in stretched cells decreasing by 1.8 and 2.4 times, respectively as compared to control levels)(Fig. 2.20). In contrast, RANTES expression was not detected in tHMC under basal conditions nor was this gene upregulated in response to cyclic strain. Modulation of the expression of the chemokine MCP-1 in these cells was also examined in view of the observation that cyclic strain is a positive modulator of MCP-1 expression in endothelial cells. Consistent results concerning the effects of cyclic strain on MCP-1 mRNA levels in tHMC proved elusive (data not shown). However, the reasons for this variability remain unclear.

IL-8 and its related C-X-C chemokines were originally thought to be predominantly chemotactic for neutrophils. A recent study has demonstrated, however, that IL-8 is a potent trigger for firm adhesion of monocytes to vascular endothelium expressing cell adhesion molecules under flow conditions. Cyclic strain has been shown to upregulate the expression of this chemokine in ECV-304 cells (section 2.3) and endothelial cells. It was somewhat unexpected, therefore, that given these responses and the putative role of glomerular monocyte/macrophage infiltration in glomerular hypertension-induced glomerulosclerosis, that exposure of tHMC to cyclic mechanical strain *negatively* modulated expression of this chemokine.

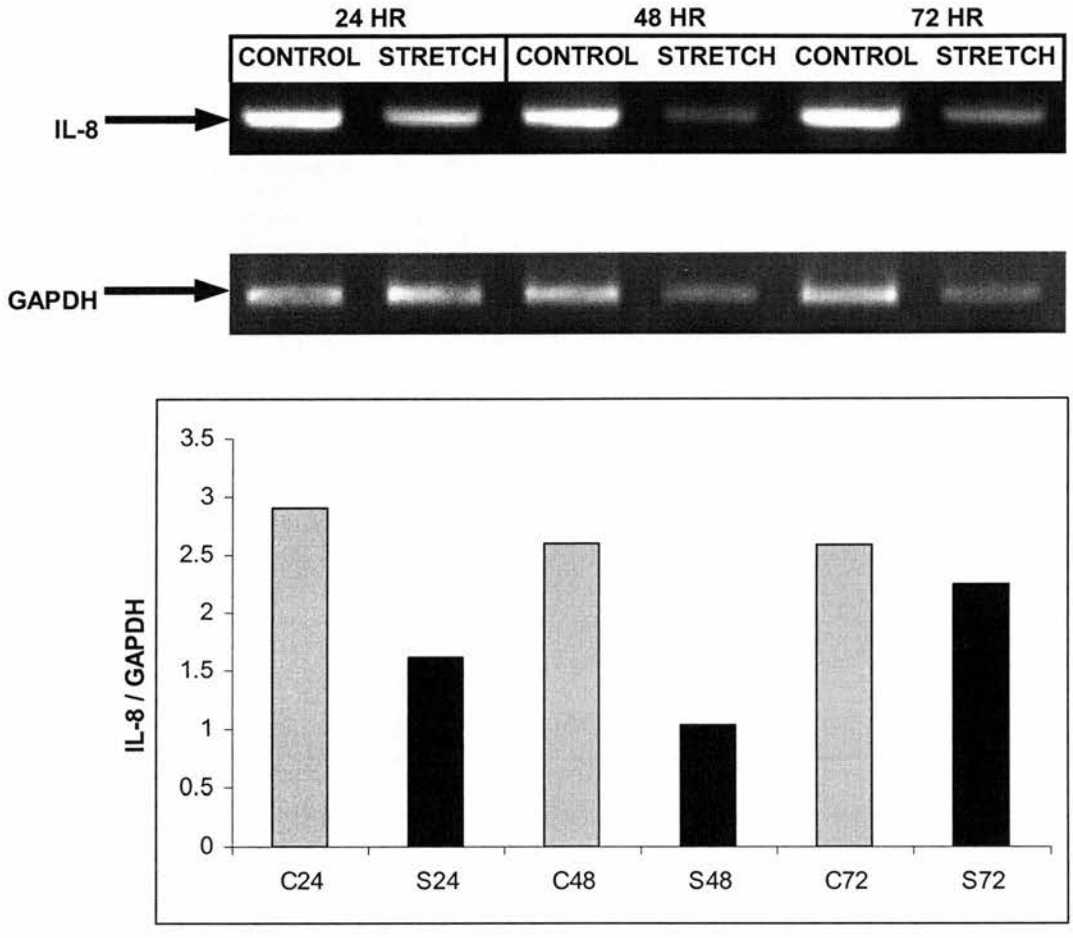


Figure 2.20 IL-8 expression in tHMC is decreased following cyclic strain.

Basal expression of IL-8 is apparent with decreases in mRNA levels for this chemokine occuring in response to 24 and 48 (but not 72) hours of cyclic strain. Result shown is representative of three separate experiments (Appendix 1B).

Mesangial cell production of IL-8 occurs in response to TNF-α and IL1-β *in vitro* and increased IL-8 expression has been demonstrated in certain experimental and human glomerulnephritides (181). Because of emerging evidence concerning the expression of chemokine receptors on many non-immune cells, it is becoming apparent that chemokines have many effects in addition to the attraction of leukocytes (section 1.2.3). IL-8, for example, has been shown to promote migration and proliferation of endothelial cells and VSMC *in vitro*, and promotes angiogenesis *in vivo*. No such effects on mesangial cells have been demonstrated however, and studies to date have focused on the chemotactic role of IL-8 in the various forms of immune-based renal diseases. The constitutive expression of this chemokine in tHMC is intriguing and it may be speculated that the down-regulation of its expression in response to cyclic strain represents a defence mechanism which protects the glomeruli from further injury by limiting monocyte infiltration. Alternatively, it may be that IL-8 exerts a specific and possibly non-chemotactic role in the kidney thereby explaining the apparently counterintuitive findings presented here.

IL-1β and IL-6 were expressed constitutively in tHMC but levels were unchanged following 24, 48 and 72 hours of cyclic mechanical strain. mRNA for the pro-inflammatory cytokines TNF-α and IFN-γ was not present in these cells under basal conditions, nor was expression of these species induced by cyclic strain at any time point studied (data not shown). These results concur with those of a previous study in which no changes in TNF-α and IL-1β expression were documented following exposure of rat mesangial cells to cyclic mechanical stretch (182). Mesangial cell production of IL-1β and IL-6 in response to pro-inflammatory stimuli has been

demonstrated *in vitro* and evidence suggests that these cytokines, acting in an autocrine way, promote the mesangial proliferation that is characteristic of various forms of glomerulonephritis (183). In addition, cytokine-modulated cytokine/chemokine/adhesion molecule expression in mesangial cells both promotes and sustains the inflammatory process in these diseases. In the context of the current study however it appears that differential responses in cytokine mRNA expression occur in tHMC exposed to cyclic mechanical stretch. Elevations in TGF-β and CTGF mRNA are not paralleled by increases in expression of the pro-inflammatory cytokines or IL-6. How these differential responses are mediated is as yet unknown. Interestingly, studies in ECV-304 cells (section 2.4) and endothelial cells (122) were also unable to demonstrate changes in expression of IL-1β, IL-6, TNF-α and IFN-γ in response to cyclic strain.

In contrast to the ample information concerning chemokines and renal injury, so far little is known regarding the expression and distribution of chemokine receptors in normal and diseased kidneys. In addition the mechanisms regulating expression of these receptors are not well-defined. It has been established, however, that in leukocytes (PMN and monocytes) and endothelial cells chemokine receptor expression can be modulated by pro-inflammatory cytokines *in vitro*. Expression of the receptors CCR1, CCR2, CCR3 and CCR5 has been detected in normal murine kidney, and levels are upregulated in animals with anti-GBM nephritis. Studies in human mesangial cells have shown that while there is no expression of the chemokine receptors CCR1-CCR8 under basal conditions, mRNA for CCR1 and CCR7 could be induced by pro-inflammatory cytokines (184).

Basal expression of the chemokine receptors CCR1, CCR2 and CXCR2 in tHMC was therefore investigated in the current study. In addition, the influence of mechanical strain on receptor expression levels was examined. In order to provide a positive control for these experiments, neutrophil and mononuclear cells were isolated from human peripheral blood using standard techniques and total RNA isolated as described in the preceding experimental section. CCR1 and CCR2 are expressed by monocyte/macrophages, while CXCR2 is expressed by neutrophils. These controls were used in the relevant PCR reactions. CCR1, CCR2 and CXCR2 were not expressed in tHMC cells, nor was mRNA for these species induced by cyclic strain (Fig 2.21).

Evidence has been presented by other investigators in our group which indicates that the strain-induced modulation of mesangial protein expression may occur independently of transcriptional and translational mechanisms. These studies have demonstrated that while levels of VCAM-1 and ICAM-1 mRNA are not increased in response to cyclic mechanical stretch, surface expression of the latter is increased significantly (as measured by FACS analysis) following exposure to 24, 48 and 72 hours of cyclic strain (185). Another study has suggested that the increase in TGF-β secretion that occurs when mesangial cells are exposed to cyclic mechanical strain in conjunction with a high glucose environment may occur via non-transcriptional mechanisms (182).

Figure 2.21 Chemokine receptor expression in tHMC cells.

mRNA for the chemokine receptors CCR1, CCR2 and CXCR2 are not expressed on tHMC mesangial cells under basal conditions or in response to 24, 48 and 72 hours of cyclic strain. Human monocytes served as positive controls for CCR1 and CCR2 while neutrophils served as CXCR2 positive control. Result shown is representative of two separate experiments.

This establishes a precedent that stretch-induced increases in protein surface expression/secretion may not in fact be due to *increased* expression of target genes. It will be necessary, therefore, to investigate whether the surface expression of leukocyte trafficking determinants such as the chemokine receptors are increased in response to cyclic mechanical strain in a manner independent of trancription/translation.

Glomerular hypertension, in addition to causing increased *mesangial* cell stretch, also results in changes in the cyclic mechanical forces experienced by the *endothelium* lining the glomerular capillaries. The effects of cyclic strain on the modulation of macrovascular endothelial gene expression *in vitro* are well-established (section 2.3), however no data exists concerning the responses of microvascular endothelial cells such as glomerular endothelial cells to this stimulus. Endothelial activation is pivotal in the pathogenesis of many disease processes and within the glomerulus these are the first obligate cells to come into contact with circulating leukocytes. The influence of cyclic mechanical stretch on the expression of leukocyte trafficking determinants and immune mediators in a rat glomerular endothelial cell line was therefore examined.

VCAM-1, but not ICAM-1, was expressed at low levels in RGEC under basal conditions. Expression levels of both adhesion molecules remained unchanged following 24 and 48 hours of cyclic strain, in contrast to TNF-α stimulation which resulted in increased mRNA levels for both species. Similarly mRNA for the chemokine GRO-α (the rat homologue of IL-8) and TGF-β, although upregulated in TNF-α-treated RGEC, were not detected under basal conditions and remained

unchanged following cyclic strain. The chemokine receptors CCR1, CCR2 and CXCR2 were not present constitutively or in response to 24 and 48 hours of mechanical strain. (Data not shown).

Thus, while the pro-inflammatory cytokine TNF-α elicits the characteristic features of endothelial activation in RGEC (increased expression of cell adhesion molecules, cytokines and chemokines), no such responses however could be demonstrated following exposure of these cells to cyclic mechanical strain. This is in contrast with our results presented here in tHMC, primary human mesangial cells and ECV-304, and indeed with that of previous investigators using macrovascular endothelial cells. The reason for the absence of strain-induced responses in RGEC is as yet uncertain. In the current study RGEC were plated onto collagen as elastin matrices did not sustain cell growth for these experiments. Prevous studies using other cell types have demonstrated that responses to mechanical stretch can be both integrin dependent and matrix specific (186). It would be of interest therefore to examine the response of RGEC grown on other types of matrix to mechanical stretch.

In summary, then, exposure of mesangial cells to cyclic mechanical strain results in increased expression of the prosclerotic mediators TGF-β and CTGF, supporting a role for this stimulus in the ECM accumulation characteristic of glomerulosclerosis. No such increases, however, occured in mesangial cell expression of cytokine, chemokine or chemokine receptors in response to mechanical strain. Indeed mRNA levels for the chemokine IL-8 appeared to decrease following exposure of cells to this stimulus. ICAM-1 however, while not increased at mRNA level, demonstrates increased mesangial cell surface expression in response to cyclic mechanical strain

indicating that stretch-induced increases in protein surface expression/secretion may not in fact be due to *increased* expression of target genes. Work is ongoing to establish whether the modulation of other mesangial cell leukocyte trafficking determinants may also occur independantly of transcriptional/translational mechanisms.

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Chapter 3

Endogenous braking signals for vascular cell activation

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Endogenous braking signals for vascular cell activation

3.1. Overview: mechanisms of the resolution of vascular disease

3.1.1. Evidence that vascular disease is reversible

Many acute inflammatory processes within the vasculature resolve without the need for specific immunosuppressive therapy. Infections, for example, once treated with appropriate antimicrobial therapy to eliminate microorganisms, are, in general, quickly acompanied by a return of tissue architecture and endothelial function to premorbid levels. The same is true of certain inflammatory conditions occuring in the absence of infection. The small vessel vasculitis, Henoch-Schonlein purpura (section 1.3.2) has an excellent prognosis with most patients recovering completely, and in some cases without the need for therapy. Similarly, in eclampsia there is no specific treatment, apart from delivery of the baby and supportive measures to treat blood pressure and seizures and while the maternal mortality continues to be significant, the multi-system abnormalities that accompany this disease quickly resolve post-partum as endothelial structure and function return to normal.

There is some evidence too for reversibility in chronic inflammatory processes such as atherosclerosis. In animal models, a low cholesterol diet results in significant plaque regression. Studies in human atherosclerosis have tended to combine a low cholesterol diet with drug therapy such as inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (statins) to lower cholesterol. It is now recognised that statins have, in addition to their cholesterol lowering properties, anti-thrombotic and anti-atherogenic effects that are independent of their lowering of

plasma-LDL (1). The regression of atherosclerosis in such trials is well-documented and would appear to be attributable to the various actions of these drugs and not endogenous anti-inflammatory mechanisms. A number of trials have attempted to assess the role of lifestyle modifications (smoking cessation, low cholesterol diet, exercise) in the progression/regression of atherosclerosis (2). Although the numbers of patients in these trials were small, these modifications, in addition to delaying progression of atherosclerosis, also resulted in significant regression as measured by coronary angiography. Removal of the injurious stimuli, therefore, appeared to promote reversal of atheroma and vessel healing, which in the absence of pharmacological intervention, is likely to be due to endogenous recovery mechanisms. In aggregate, these observations in acute and chronic vascular diseases suggest the existence of endogenous systems which *actively* restore vascular tone, thrombogenicity and adhesiveness for leukocytes to normal.

3.1.2. Mechanisms of resolution

It is now apparent that the resolution phase of such inflammatory processes, like the initiation phase, is a dynamic process in which a large number of anti-inflammatory molecules are elaborated under well-ordered programs that act as "braking signals" for leukocyte recruitment and subsequent tissue injury and promote healing and repair.

These so-called "defence" molecules counteract the inflammatory response at all levels, extracellular, cell surface and intracellular. Some have very specific targets e.g. cytokine inhibitors, proteinase inhibitors, complement regulatory proteins, anti-thrombotic molecules, antioxidants and cyclin kinase inhibitors. Conversely

mediators such as the anti-inflammatory cytokines, anti-inflammatory eicosanoids, glucocorticoids, heparin, adenosine and nitric oxide have a wide range of targets. Some of these molecules are expressed constitutively, while others are inducible in response to a local inflammatory milieu. These various mediators and their mechanisms of action are reviewed extensively elsewhere (3). Of particular relevance to the work described here are the lipoxins, a group of rapid-acting anti-inflammatory eicosanoids will be discussed in detail in section 3.2. In addition, the anti-inflammatory cytokines and endogenous inhibitors of cytokine bioactivity are reviewed in section 3.5. Particular emphasis will be placed on a recently identified family of proteins, the suppressors of cytokine signalling (SOCS) which effectively act as cytokine inhibitors. The actions of the SOCS proteins and the evidence supporting a role for them in the modulation of cytokine bioactivity in health and disease are examined in section 3.6.

In summary then, the observation that many inflammatory reactions resolve spontaneously indicates the presence of endogenous 'braking systems' that 'turn-off' leukocyte-mediated inflammation and limit tissue injury. Defining the molecular basis for these various anti-inflammatory mediators is critical to the further understanding of the inflammatory response in addition to the possibility of exploiting their activities for therapeutic gain.

3.2. Lipoxins: putative braking signals for acute polymorphonuclear leukocyte (PMN)-mediated vascular injury

Recruitment of polymorphonuclear leukocytes (PMN) from blood to an extravascular inflammatory focus is an essential feature of host defense against bacterial and fungal infection. PMN recruitment from blood vessel lumen into target tissue occurs via the classical paradigm of leukocyte trafficking described in previous sections. Having reached the target tissue, PMN eliminate invading microorganisms by phagocytosis and subsequent release of free radicals and lytic enzymes into phagolysomes. Inappropriate leukocyte trafficking and activation, such as occurs in the various phenotypes described in section 1.3, can result in extensive tissue damage and organ dysfunction in the absence of infection. Many inflammatory reactions resolve spontaneously suggesting that endogenous 'braking systems' exist that 'turn-off' leukocyte-mediated inflammation and limit tissue injury (4). Evidence is reviewed that the lipoxins serve this purpose.

3.2.1. Lipoxin biosynthesis *in vitro*

The lipoxins (LX) are eicosanoids that carry a trihydroxytetraene structure and are structurally and functionally distinct from other families of arachidonic acid-derived bioactive products. In suspensions of activated granulocytes *in vitro*, the sequential actions of 15- and 5-lipoxygenase on arachidonic acid yields 5,6-epoxytetraene, an unstable epoxide intermediate, which is converted by epoxide hydrolases to the two major bioactive lipoxins LXA_4 and LXB_4 (5, 6). The latter are positional isomers: the structure of LXA_4 being 5*S*, 6*R*,15*S*-trihydroxy-7,9,13 trans-11-cis-eicosatetraenoic

acid and the structure of LXB_4 being 5*S*,14*R*,I5*S* hydroxy-6,10,12-trans-8-cis-eicosatetraenoic acid (7) (Fig. 3.1).

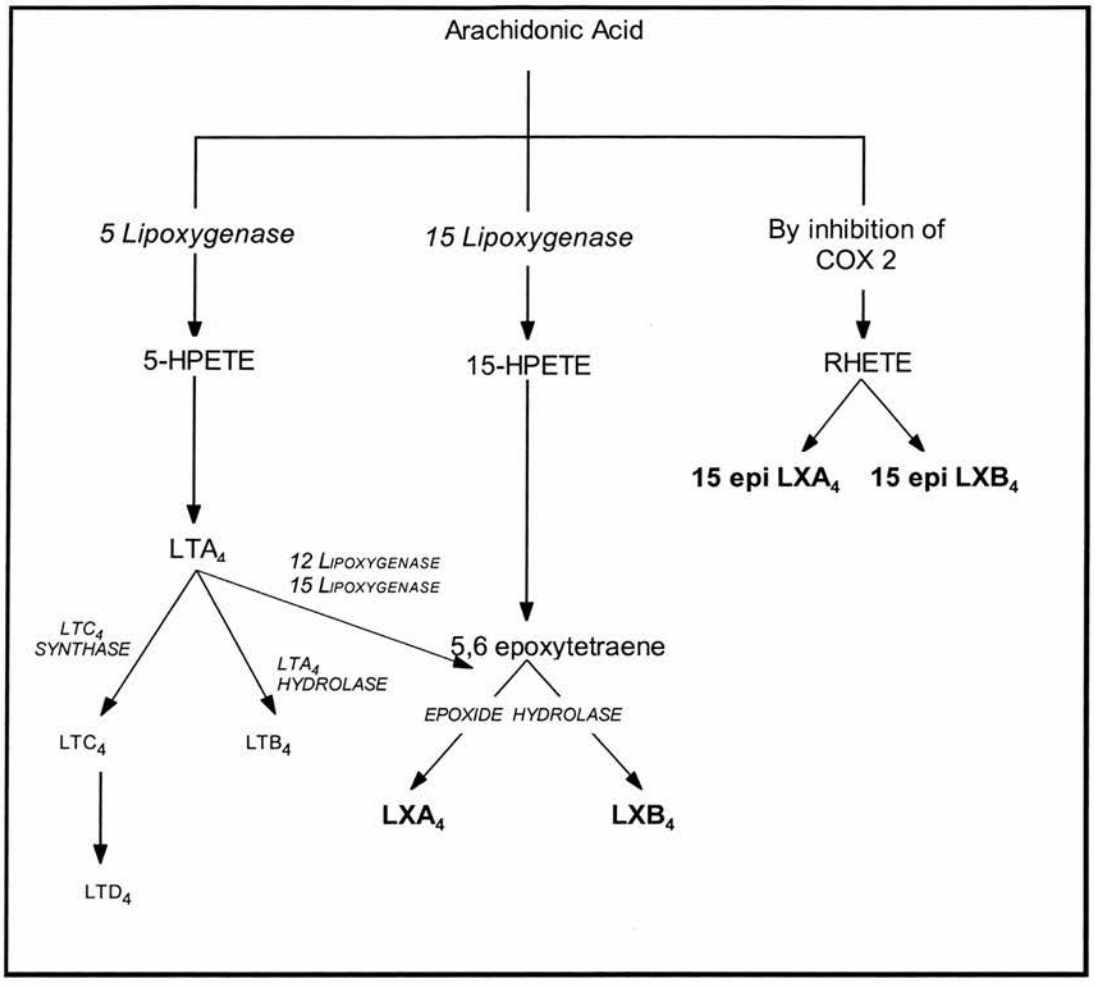


Figure 3.1 Summary of major pathways for leukotriene and lipoxin biosynthesis.

Abbreviations:

HETE-	Hydroxyeicosatetrenoic acid
COX 2-	Cyclooxygenase-2

Lipoxin biosynthesis is augmented greatly *in vitro* if granulocytes are activated in the presence of platelets (8-12). In this setting, platelets convert PMN-derived LTA_4 to 5,6-epoxytetraene through the action of platelet 12-lipoxygenase. The latter enzyme functions as a 15-lipoxygenase (LX synthase) when its substrate is LTA_4. Intriguingly, aspirin triggers production of lipoxin *R*-epimers during coincubations of

PMN and cytokine-activated endothelial cells *in vitro* (13, 14). When acetylated by aspirin, cyclooxygenase 2 (COX-2) retains the enzymatic capacity to convert arachidonic acid to 15(*R*)-HETE. During cell-cell interactions, some endothelial-derived 15(*R*)-HETE is converted by PMN 5-lipoxygenase to either 15 epi-LXA_4 or 15-epi LXB_4 which display properties similar to native lipoxins (14). (see below).

Lipoxin production is boosted *in vitro* by several mechanisms that may be relevant to lipoxin generation in human disease. Cell-cell adhesion through P-selectin promotes transcellular biosynthesis of lipoxins during PMN-platelet interaction probably by enhancing the transfer of arachidonate intermediates between different cell-types and by priming lipoxygenase pathways (15, 16). Disruption of platelet cell membranes, which occurs over time within an inflammatory or thrombotic microenvironment, also enhances lipoxin production (10). The balance of lipoxin:leukotriene generation during PMN-platelet interaction is also influenced by local redox potential. Specifically, a fall in cellular levels of reduced glutathione, as occurs during PMN-mediated or ischaemic injury, augments lipoxin levels at the expense of leukotriene generation (9).

Finally, '*in-vitro*' experiments predict that cytokines are important local regulators of lipoxin biosynthesis. During PMN-platelets interactions, transcellular lipoxin generation is also boosted by GM-CSF and PDGF. IL-4 and IL-13 induce 15-lipoxygenase expression in alveolar macrophages, granulocyte, monocytes and epithelial cells, activities that may promote transcellular lipoxin generation (17, 18). Of note, these T_H-2 derived cytokines have been implicated as negative regulators of the inflammatory response.

The pathways for lipoxin metabolism are still being defined. Lipoxins can be metabolised rapidly *in vitro* through either dehydrogenation or ω oxidation (19, 20). It is likely that the former represents the major pathway for lipoxin degradation *in vivo*. With a view to probing the bioactions of lipoxins further and to possible drug design, lipoxin analogues have been designed and synthesized which are resistant to degradation and retain the biological activity of native lipoxins (21, 22). These have shown promising results in preliminary studies in experimental animal models of acute inflammation.

3.2.2. Lipoxin generation *in vivo*

Lipoxins have been detected in tissue, blood or body fluids in a variety of human diseases including asthma, glomerulonephritis, rheumatoid arthritis, psoriasis, pneumonia, post-coronary angioplasty, sarcoidosis, and allergic rhinitis (7, 23, 24). Interestingly, platelet-PMN transcellular pathways appear to be an important source of lipoxin generation during renal glomerular inflammation (25) and after angioplasty (24) *in vivo* confirming predictions from *in vitro* experiments. It is likely that PMN also interact with other cell-types to generate lipoxins as inflammation progresses and cytokines induce 15-lipoxygenase expression in resident tissue cells (see above).

3.2.3. Lipoxins: potent modulators of leukocyte-endothelial cell adhesion

The spectrum of biological activities of lipoxins is still expanding. Lipoxins attenuate many pro-inflammatory effects of leukotrienes and other mediators *in vitro*. In particular, they inhibit PMN-endothelial cell adhesion, a critical step for PMN

recruitment *in vivo* (26). The various leukocyte-endothelial adhesion molecules and their corresponding ligands are discussed in detail in section 1.2.2.

Leukotrienes are established proinflammatory mediators and modulate PMN trafficking, vascular permeability and blood flow (27). LTB_4 is a potent stimulus for PMN chemotaxis and PMN-endothelial cell adhesion supported by CD11/CD18 integrins. LTC_4 and LTD_4, in contrast to LTB_4, show limited bioactivity towards human PMN, but act on smooth muscle cells to induce vasoconstriction and bronchoconstriction. In addition, LTC_4 and LTD_4 trigger PMN-endothelial cell adhesion *in vitro* by inducing formation of endothelial cell mediators that promote PMN adhesion (e.g. platelet-activating factor) and by stimulating mobilization of P-selectin to the cell surface (26, 28, 29). Mice transgenic for the LTB_4 receptor have demonstrated that over-expression of this receptor dramatically increases PMN trafficking both to skin microabscesses and to lungs in a hindlimb tourniquet model of second organ reperfusion injury (30). This was associated with selective increases in the expression of 5-lipoxygenase and its product 5*S*-HETE in inflammatory exudates, leading the authors to speculate that receptor overexpression amplifies pro-inflammatory circuits. In contrast, mice deficient in 5-lipoxygenase showed diminished PMN accumulation in lungs, indicating the importance of both the LTB_4 receptor and the 5-lipoxygenase enzyme in reperfusion-initiatiated PMN trafficking to remote organs.

Lipoxins antagonise many of the actions of leukotrienes *in vitro* (see Table 3.1). LXA_4 and LXB_4 themselves do not provoke chemotaxis, adhesion, degranulation or free radical generation at biologically relevant concentrations (5). In contrast, LXA_4

and LXB_4 inhibit LTB_4-induced PMN chemotaxis and PMN-endothelial cell adhesion supported by CD11/CD18 integrin (26). LXA_4 and LXB_4 also attenuate LTC_4- and LTD_4 -triggered endothelial hyperadhesiveness for PMN, at least in part by inhibiting mobilisation of P-selectin from Weibel-Palade bodies to the endothelial cell surface (26)(Fig 3.2). In addition, LXA_4 can attenuate the contraction of smooth muscle and mesangial cells produced by LTC_4 and LTD_4-induced vasoconstriction (31, 32).

Bioactivity modulated by lipoxins	Lipoxin	Stimulus	Ref
Leukocytes			
PMN chemotaxis	LXA_4, LXB_4	LTB_4, fMLP	(33)
PMN endothelial cell adhesion	LXA_4, LXB_4	LTB_4 (not C5a, fMLP)	(26)
PMN migration across endothelium	LXA_4, LXB_4	LTB_4 (not C5a, fMLP)	(26)
PMN migration across epithelium (basolateral-to-apical only)	LXA_4, 11-trans-LXA_4 (not LXB_4)	fMLP	(34)
PMN homotypic aggregation	LXA_4	fMLP	(35)
Upregulation of CD11/CD18	LXA_4	fMLP	(35)
PMN margination and diapedesis	LXA_4	Topical LTB_4	(34)
PMN infiltration of glomeruli	LXA_4	Immune complex GN	(15)
Endothelial Cells			
P-selectin mobilization	LXA_4, LXB_4	LTC_3, LTD_4 (not histamine, thrombin)	(26)
Increased adhesiveness for PMN	LXA_4, LXB_4	LTC_4, LTD_4 (not histamine, thrombin)	(26)
Mesangial Cells			
Mesangial Cell adhesiveness	LXA_4	LTD_4	(27)
Mesangial Cell contractility	LXA_4	LTD_4	(36)

Table 3.1. Some actions of lipoxins.

The 'anti-inflammatory' actions of lipoxins are not confined to leukotriene-triggered responses. LXA_4 also antagonises fMLP-induced PMN chemotaxis (33), eosinophil

chemotaxis induced by platelet activating factor, (37) and IL-8 release from TNFα primed colonic cell lines (38). In addition, lipoxins attenuate PMN transmigration across epithelial monolayers activated by TNF α (34). It has also been demonstrated that both LXA_4 and aspirin triggered 15-epi-LXA_4 inhibit the release of superoxide anion and IL-1β from TNF-α-stimulated neutrophils *in vitro* (39). A recent study from our group shows that LXA_4 stimulates tissue factor (TF) activity in monocytes and endothelial cells *in vitro* although the *in vivo* importance of LXA_4 as a modulator of TF-related vascular events during inflammation and thrombosis is still unclear (40).

In keeping with *in-vitro* observations, LXA_4 inhibits LTB_4-induced PMN margination and diapedesis in hamster cheek pouch model of the microcirculation (41). In the mouse ear model of inflammation, epi-lipoxins and stable lipoxin analogues inhibit PMN infiltration evoked by topical application of LTB_4 (14). Furthermore in the inflammatory murine air pouch model, a stable analogue of lipoxin, 15R/S-methyl-LXA_4, inhibits neutrophil recruitment. Interestingly this is with associated a decline in MIP-2 and IL1β and an increase in IL-4 production in the pouch exudates (30). In mice transgenic for the LTB_4 receptor, topical application of lipoxin is protective in acute dermal inflammation, while intravenous injection of aspirin-triggered 15-epi-lipoxin diminishes PMN trafficking in the lung in a model of second organ reperfusion injury (39). Exposure of PMN to LXA_4 *ex vivo* attenuates their recruitment to inflamed renal glomeruli in the Con A/Ferritin model of glomerulonephritis (15). Finally, defective LXA_4 biosynthesis is associated with exaggerated PMN infiltration in experimental immune complex-mediated glomerulonephritis in P-selectin knockout mice (41).

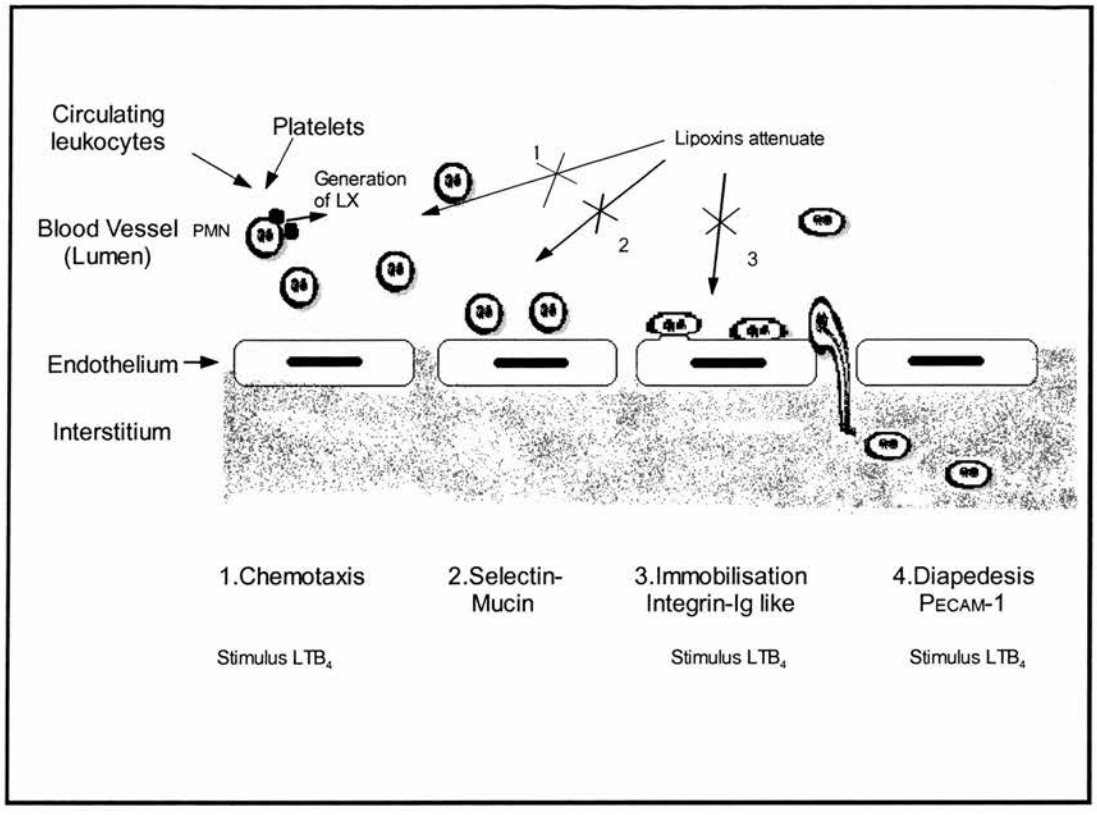


Figure 3.2. Modulation of leukocyte trafficking by lipoxins.

Lipoxins attenuate (1) Chemotaxis (2) Selectin-mediated adhesion (3) Integrin-mediated immobilisation.

3.2.4. Cellular mechanisms of lipoxin actions

The molecular basis for these lipoxin-evoked responses is still being elucidated. PMN, monocytes, gastrointestinal epithelial cells, renal mesangial cells and endothelia express high affinity binding sites for lipoxins (35, 38, 42). The cloning and characterisation of these lipoxin receptors suggest that many are members of the seven transmembrane spanning superfamily of G-protein coupled receptors. cDNA cloning and subsequent sequence analysis of the human myeloid LXA_4 receptor reveals it to be homologous to members of the chemokine receptor superfamily

which also includes the fMLP receptor and the recently cloned LTB_4 receptor (14, 38). This PMN receptor has also been cloned from monocyte and epithelial cDNA libraries (14).

Engagement of the monocyte and PMN LXA_4 receptor causes mobilisation of intracellular calcium, activation of phospholipases A_2, C and D, and arachidonic acid release. These responses are blunted by pertussis toxin (PTX) further supporting involvement of heptahelical G protein-coupled receptors (35, 42, 43). Downstream of these signalling pathways are the mitogen activated protein (MAP) kinase cascades. In response to extracellular stimuli, MAP kinases regulate the transcriptional activity of several transcription factors, thereby initiating the expression of a variety of immediate and delayed early response genes. Data from our laboratory has demonstrated that in mesangial cells, LXA_4 stimulates phosphorylation and activation of specific MAP kinases in a PTX insensitive manner; in contrast, LTD_4 activation of these pathways appears to be PTX sensitive (44). The structure and function of the mesangial cell and endothelial receptors are under investigation. It has been suggested that LXA_4 attenuates the proinflammatory effects of LTD_4 in mesangial cells by acting as a partial agonist at an LTD_4 binding site (36). To date the LXB_4 receptor has not been cloned. Pharmacological evidence suggests that it is distinct from the LXA_4 receptor (7).

In aggregate, these observations suggest that the leukotrienes and lipoxins are notably distinct from other eicosanoids both in terms of their functional activities and their cell signalling pathways. The homology of lipoxin receptors to chemokine receptors is particularly interesting and suggests that the lipoxin/leukotriene

pathways may have evolved principally to modulate granulocyte trafficking during host defence.

3.3. Mitogen-activated protein (MAP) kinases

Mitogen-activated protein kinases are serine-threonine protein kinases that, in response to a diverse array of extracellular signals, initiate a variety of cell-signalling outcomes. Five mammalian MAP kinase families have been identified to date, each with distinct biological functions; extracellular signal related kinase (ERK) 1/2, p38 MAPK, c-*Jun*-N-terminal kinase (JNK), ERK3/4 and ERK5 (45). A common feature of all MAP kinase isoforms is the requirement for phosphorylation of both threonine and tyrosine regulatory sites by a specific upstream protein kinase for activation. The basic assembly of MAP kinase pathways is a three-component module comprising three kinases which establish a sequential activation pathway: MAP kinase kinase kinase (MKKK), MAP kinase kinase (MKK) and MAP kinase. In response to stimuli, this sequence is activated such that the ultimate activation of the tertiary MAP kinase results in the phosphorylation of either stimulatory or inhibitory transcription factor sites, thereby modulating the expression of a variety of immediate and delayed early response genes. This, in addition to the phosphorylation and regulation of cytosolic and nuclear targets, is critical in producing the various cellular responses which typify MAP kinase activation including proliferation, differentiation, adaptation to environmental stress and apoptosis (Fig 3.3).

3.3.1. ERK Pathway

There are five MAP kinases defined as ERK, however amino acid sequence comparisons indicate that these proteins belong to different families of MAP kinases. Of this collective group known as ERK, ERK 1 and ERK 2 (44- and 42-kDa isoforms respectively) are the most extensively studied. The other ERK are less well characterised: these include ERK 3 which is localised in the nucleus and is activated

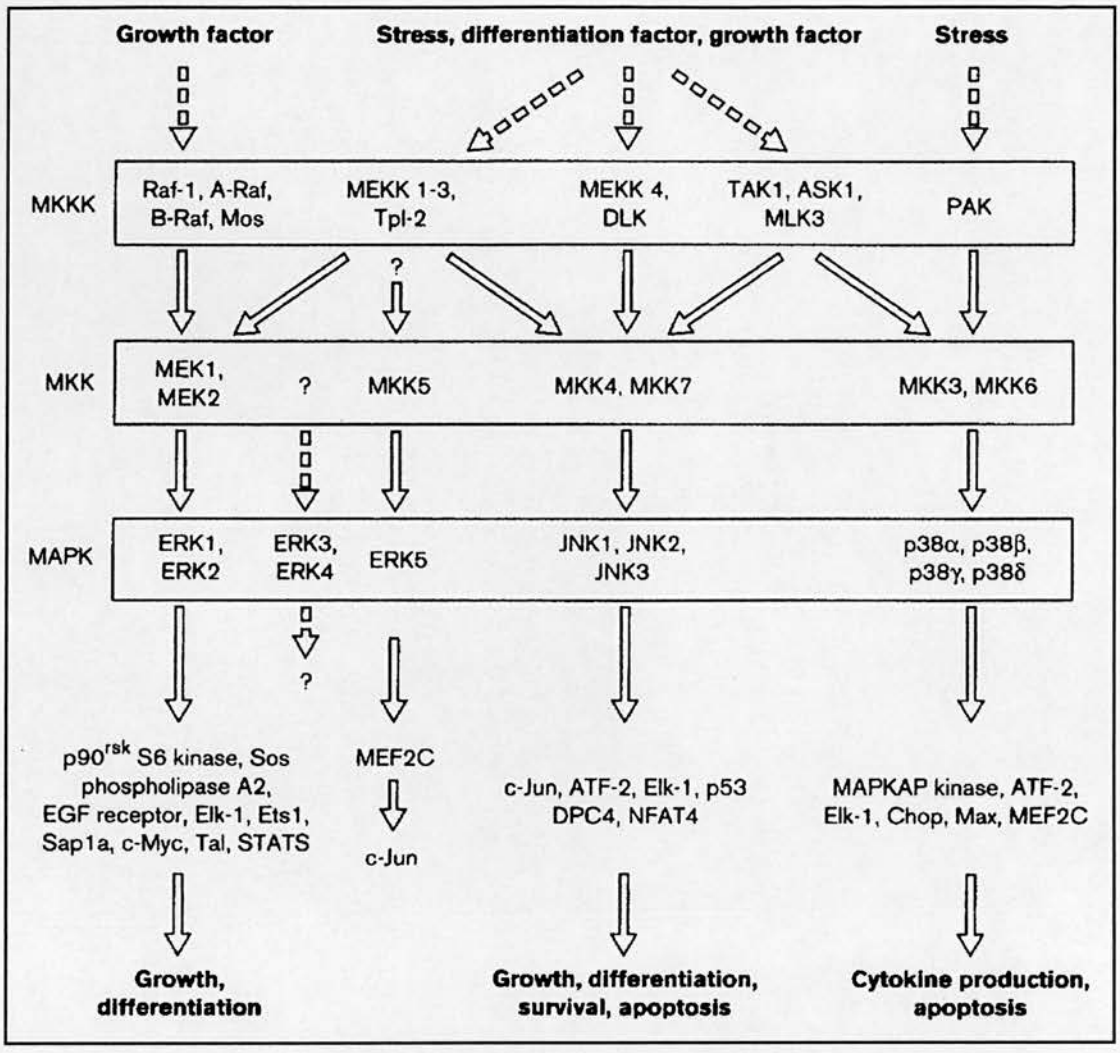


Figure 3.3. Overview MAP Kinase pathways (46).

by PKC isoforms, ERK 4 which is activated in response to nerve growth factor (NGF) and EGF via a Ras-dependent pathway and the least known pathway, ERK 5, which in response to oxidative stress/hyperosmolality/serum translocates from the cytosol to the nucleus where it activates the transcription factor MEF2C which induces expression of c-Jun (45). For the remainder of this discussion, however, the term ERK will refer to ERK 1 and 2.

Many different receptor types activate the ERK pathway. These include receptor tyrosine kinases (which bind growth factors), cytokine receptors, G-protein coupled receptors and the engagement and clustering of integrins. Ligation of many receptors leads to activation of ERK through the activation of Ras (47). Ras in the GTP-bound form binds to Raf 1 which functions as the activated MKKK in this pathway. $MKKK^{Raf1}$ in turn activates the MKKs, MEK 1 and 2, although these appear to be differentially activated depending on cell type studied. These kinases then phosphorylate and activate ERK. There have been some reports that ERK activation can occur in the absence of Raf activation suggesting that other MKKK may function in the ERK signalling modules. Upon activation, ERK phosphorylates substrate proteins on serine or threonine residues within a proline-directed motif. The cytoplasmic proteins known to be substrates for ERK include the S6 kinase p90rsk, phospholipase A_2 and several microtubule associated proteins (48). Following activation ERK is also capable of translocation to the nucleus where it can result in the phosphorylation of several transcription factors including Elk-1, Ets-1 c-Myc, c-Jun, Sap 1a, Tal and signal transducer and activator of transcription (STAT) proteins. Given these many diverse substrates, it is not surprising that the biological effects of ERK activation are wide ranging. In cell culture systems however there is a good evidence to support a role for ERK in cell proliferation, differentiation, regulation of cell cycle activity and protection from apoptosis.

3.3.2. JNK pathway

The c-Jun-N-terminal kinase (JNK) is so named because of its ability to regulate c-*Jun* transcriptional activity by phosphorylation of the N-terminal activating domain (whereas ERK phosphorylates the inhibitory C-terminal site). JNK is activated by

extracellular stress (UV-light, heat shock, osmolarity), cytokines (TNF-α, IL-1) or growth factors (45, 46). 10 different isoforms exist but to date a definitive analysis of their expression patterns *in vivo* has not been carried out. While the pathways that lead from cell stress to activation of this cascade remain largely uncharacterised, the JNK response to extracellular ligands has been studied extensively. Activation of this pathway can occur in response to stimulation of a variety of different receptor types including the TNF receptor family, G-protein coupled receptors, tyrosine kinase receptors and cytokine receptors. MKK 4 and MKK7 have been implicated in the phosphorylation and activation of JNK. These kinases are in turn activated by several MKKK (up to 10 have been demonstrated to act in the JNK signalling module). Other signalling proteins that act as upstream activators of the JNK pathway are the small GTP-binding proteins of the Rho and Rac family. In studies, for example, in which mutation of the native genes results in constitutively active protein varients of Rac and Cdc 42, JNK activation ensues. The JNK substrates identified to date are exclusively transcription factors (in contrast to ERK which has substrates outside the nucleus), and include c-Jun, ATF-2, Elk-1, p53, DPC4 and NFAT4. JNK activation results in diverse responses depending on cell type, but evidence suggests that this signalling pathway may exert anti-proliferative and pro-apoptotic effects in addition to promoting T-cell activation.

3.3.3. p38 MAP kinase

This family consists of at least four different homologous proteins – p38 α, β, γ and δ (45). Once again p38 MAP kinase activation occurs in response to a variety of stimuli – cellular stress (UV irradiation, osmotic shock, LPS), cytokine receptor binding and G protein coupled receptors, and a similar ordered activation module

exists. A number of MKKK and MKK have been implicated in the subsequent activation of p38 MAP kinase. (Fig 3.3).

Interestingly the MKKK Pak (also known as p21-activated kinase) can be activated by Cdc 42 and Rac, G proteins which are also implicated in the upstream signalling of the JNK pathway. Activated p38 MAP kinase phosphorylates a variety of substrates. These include the transcription factors ATF2, Elk-1, Chop and Max. In addition, p38 activation can result in the phosphorylation of small heat-shock proteins. This MAP kinase pathway is important in the production of cytokines by haematopoietic cells, IL-2 mediated cellular proliferation and promoting apoptosis.

3.4. Differential effects of lipoxins on endothelial and mesangial cell MAP kinase pathways

3.4.1. Introduction

The activation of the endothelium and its subsequent interaction with circulating blood cells is a pivotal event in the pathophysiology of inflammation, allograft rejection, atherosclerosis and thrombosis. The robust acute inflammatory response which is initiated by endothelium:PMN interaction is typically self-limiting and tissue architecture and function normally return to premorbid levels. These observations suggest that the resolution phase of inflammation is a dynamic process and that endogenous anti-inflammatory agents are elaborated that can act as "braking signals" for leukocyte recruitment and subsequent tissue injury. The actions of lipoxins in this regard are discussed in the preceding section (2.4). In brief, lipoxins attenuate many pro-inflammatory effects of leukotrienes (and indeed other mediators) *in vitro*. Moreover *in vivo*, they inhibit PMN-endothelial cell adhesion, a critical step for PMN recruitment. Although, these effects appear to be regulated via high affinity G protein coupled receptors, the more downstream cell signalling events mediating these novel bioactions remain undefined. The neutrophil LXA_4 receptor has been previously shown to be coupled to phospholipase activation and increases in intracellular calcium levels (35, 42). In mesangial cells, it has been suggested that LXA_4 attenuates the proinflammatory effects of LTD_4 by acting as a partial agonist at an LTD_4 binding site (36) but it is not known whether this is the case for endothelial cells. The structure of the endothelial cell LXA_4 receptor and the sequence of events following its engagement have not been elucidated. The aim of this study therefore was to characterise the signal transduction events triggered by activation of

endothelial lipoxin and leukotriene receptors, and specifically to examine whether MAP kinase activation (section 3.3) occurred in response to these stimuli.

3.4.2. Materials and Methods

Cell Culture

ECV 304 cells have been previously characterised (49) and were obtained from the EATCC (Cat no. 92091712). All cell culture reagents were obtained from Gibco Life Technologies (Paisley, Scotland) unless otherwise stated. These cells were maintained in M199 medium (Sigma, Dorset, England), supplemented with 1% glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% heat-inactivated foetal calf serum. Cells were grown in 100 mm dishes and growth media was replaced with M199, containing 0.5% FCS 48 hours prior to stimulation.

Cell Treatments

All materials were obtained from Sigma (Dorset, UK) unless otherwise stated. The cells were stimulated with LTD_4, LXA_4, (Cascade, USA) alone or in combination at 10^{-7} M for the times indicated. Stimulations with phorbol l2-myristate-13-acetate (PMA) (100 nM) and anisomycin (100 nM) were carried out in the relevant experiments. The appropriate vehicle controls were included in all experiments. Following stimulation the media was aspirated and the cells washed with ice cold Dulbecco's PBS (without calcium or magnesium). Cells were lysed in 500 μl of ice cold whole cell extract buffer (20 mM HEPES, 75 mM NaCl, 2.5 mM $MgCl_2$, 0.1 mM EDTA, 0.05% Triton X-100, 0.5 mM dithiothreitol, 20 mM β-glycerophosphate, 0.1 mM sodium orthovanadate, 2 μg/ml leupeptin, 100 μg/ml phenylmethylsulfoflyl fluoride). Following incubation on ice for 5 minutes, lysates were scraped from each

dish and transferred to microfuge tubes. These samples were left at 4^0C for a further 10 minutes and then centrifuged at 4^0C at 20,000g for 10 minutes. Supernatants were removed and stored at -20^0C for further analysis.

Protein Determination

The protein concentration in each sample was determined using a Bradford Assay (Bio-Rad, Hercules, CA). The absorbance of each sample was measured at a wavelength of 595 nm (Ultraspec 3000, Pharmacia Biotech. Inc., Almeda, CA). A standard curve was prepared using bovine serum albumin standards (0. 1, 2, 4, 6, 8, 10 μg/ml) and this was used to estimate the concentration of protein present in a sample aliquot.

Western Blot Analysis

Lysates standardised for protein were mixed with 5X Laemmli buffer and heated for 5 minutes at 95^0C. Typically 40 μg protein/sample was subjected to SDS-PAGE using 4 and 10% acrylamide for stacking and resolving gels respectively. Protein was electrotransferred to polyvinylidene difluoride (PVDF) membranes at 40 V for 4 hours or 10 V overnight. To confirm equivalence of both protein loading and transfer, a strip of membrane was cut well above the expected location of the phosphorylated proteins. This membrane was stained using Coomassie stain for 2 minutes, followed by Coomassie destain (50% methanol, 10% acetic acid) for 30 minutes and dried. The remaining membrane containing the proteins of interest was washed with TTBS (Tris Buffered Saline, pH 7.6, 0.1% Tween-20) and blocked for 1 hour in blocking buffer (TTBS, pH 7.6, 5% nonfat dry milk [w/v]). An accepted method of detecting phosphorylated, and hence active, MAP kinases involves

immunoblotting using anti-phospho-MAP kinase antibodies. For the purposes of this study, the membranes were probed with a primary antibody specific for either phosphorylated ERK, p38 MAPK or JNK (New England Biolabs, MA, USA). The membranes were incubated with the primary antibody at a dilution of 1:1000 in TTBS, pH 7.6, 5% Bovine Serum Albumin (w/v) overnight at 4^0C. Following three five minute washes in TTBS, incubation was carried out with goat anti-rabbit alkaline phosphatase (Bio-Rad, Hercules, CA), at a dilution of 1:3000 in TTBS, pH 7.4. 3% nonfat dry milk (w/v) for 1 hour. This was followed by two further 5 minute washes in TTBS and a final 5 minute wash with TBS. The blots were developed using 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) liquid substrate system.

3.4.3. Results and discussion

ECV 304 cells, a spontaneously transformed cell line isolated from human umbilical vein endothelial cells, were chosen for use in these studies. These cells appeared to be a well-established *in vitro* model system, whose stable phenotype was ideally suited for studies of endothelial cell signalling. The subsequent identification of these cells as T24 cells (human bladder carcinoma cell line) is discussed in detail in section 2.4. This may in part explain the problems pertaining to the experiments about to be described. In addition, because of the epithelial nature of these cells, it is not possible to directly correlate between any responses demonstrated here and those occuring in endothelial cells.

Experiments focused initially on studies into the putative activation of the MAP kinases ERK, JNK and p38 MAPK in ECV-304 cells in response to lipoxin and

leukotriene stimulation. To this end, phosphorylation (i.e. activation) of these proteins was detected by means of Western blotting using phospho-specific antibodies. Attempts were made to establish the temporal kinetics and dose dependence of both lipoxin A_4 (LXA_4) and leukotriene D_4 (LTD_4)-dependent ERK, JNK and p38 MAPK activation. Initial time course experiments were carried out at 10^{-7} M for both ligands since prior studies indicated that these were likely to be optimal doses for both LXA_4 and LTD_4. LTD_4 for example provokes a rapid increase in endothelial P-selectin at 10^{-7} M, an effect which is maximally inhibited by lipoxin at a concentration of 10^{-7} M (26).

Activation of ERK in ECV-304 cells in response to LTD_4 treatments at 10^{-7} M for 2, 5, 10, 20 and 30 minutes was examined (Fig.3.4). Fully phosphorylated and non phoshorylated MAP kinase control proteins (NEB) confirmed that primary antibody specifically detected the modified MAPK (Lanes 1 and 2). Cells treated with PMA (10^{-7} M, 15 minutes), a known activator of ERK, served as a positive control (Lane 6). ERK activation occurred under basal conditions and at all time points studied. Equivalence of both protein loading and transfer was confirmed in all experiments (data not shown). The presence of activated ERK under basal conditions was such that even PMA, a known potent activator of ERK, appeared to give only a moderate response. It is not surprising therefore that the effects of a (presumably) weaker stimulus such as LTD_4 on ERK phosphorylation were undetectible. Indeed, in the current study, it was not possible to demonstrate increases in ERK activation in response to LTD_4 over the time course studied.

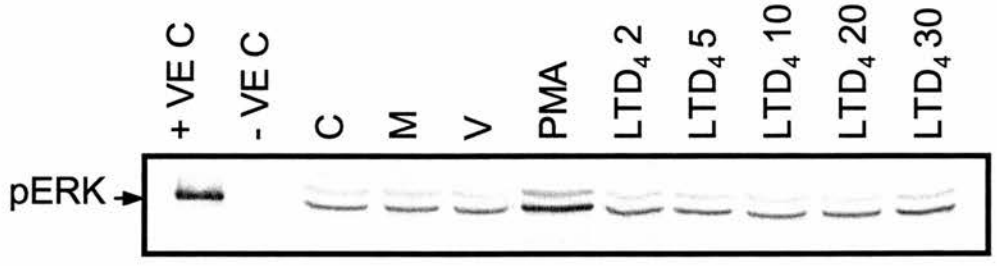


Figure 3.4. ERK activation in ECV-304 cells occurred under basal conditions and at all time points studied.

ERK activation in ECV-304 cells in response to LTD_4 (10^{-7} M) for 2, 5, 10, 20 and 30 minutes. Fully phosphorylated and non phoshorylated MAP kinase control proteins (NEB) confirmed that primary antibody specifically detected the modified MAPK (Lanes 1 and 2). Cells treated with PMA (10^{-7} M, 15 minutes), a known activator of ERK, served as a positive control (Lane 6); vehicle control was ethanol (0.1% v/v). ERK activation occurred under basal conditions and at all time points studied. Lanes 3 and 4 refer to the negative control (C=control) and cells in which extra care was taken to avoid excessive movement of the medium (M=medium), respectively.

Figure 3.5 ERK activation in ECV-304 cells in response to LTD_4 at concentations of 10^{-11} M to 10^{-6}

High constitutive levels of phosphorylated ERK were apparent, with no obvious increase in response to LTD_4 at any dose tested. PMA and vehicle (0.1% v/v ethanol) controls were included as before.

A number of practical steps were taken in an effort to reduce basal ERK activation. There had been concern that ERK activation might be sensitive to perturbation of the cells during handling and addition of stimuli. The levels of phosphorylated ERK were therefore measured in cells in which extra care had been taken to avoid excessive movement and from which no medium was removed or stimulant added. (Lane 3, M=medium). There was however no apparent decrease in the basal levels in these cells when compared to those from whom medium had been removed and readded (Lane 4, C=control) or the vehicle control (Lane 5, V=vehicle, ethanol 0.1% [v/v]). These stimulations were repeated on a number of occasions with broadly similar results. (Figure 3.4 shows a representative experiment). Attempts were made to establish a dose-response curve for ERK activation in response to LTD_4 at concentrations ranging from 10^{-11} M to 10^{-6} M for 5 minutes (Fig. 3.5). Once again, these were frustrated by high constitutive levels of phosphorylated ERK, with no apparent increase in response to LTD_4 at any dose tested. Treatment of ECV-304 with LXA_4 at 10^{-7} M over the same time course produced similar results (data not shown).

The results outlined are broadly similar with respect to p38 MAPK and JNK activation over the same time course. Anisomycin (10^{-7} M, 15 minutes), a known activator of these kinases, served as a positive control in these experiments. Total cell extracts from control and anisomycin C-6 glioma cells treated served as positive and negative controls respectively, for phosphorylated and hence activated p38 MAPK (New England Biolabs, MA, USA). Experiments were performed a number of times and representative blots are shown in figures 3.6 and 3.7. Once again, high constitutive levels of the active kinases were apparent with no increases by LTD_4.

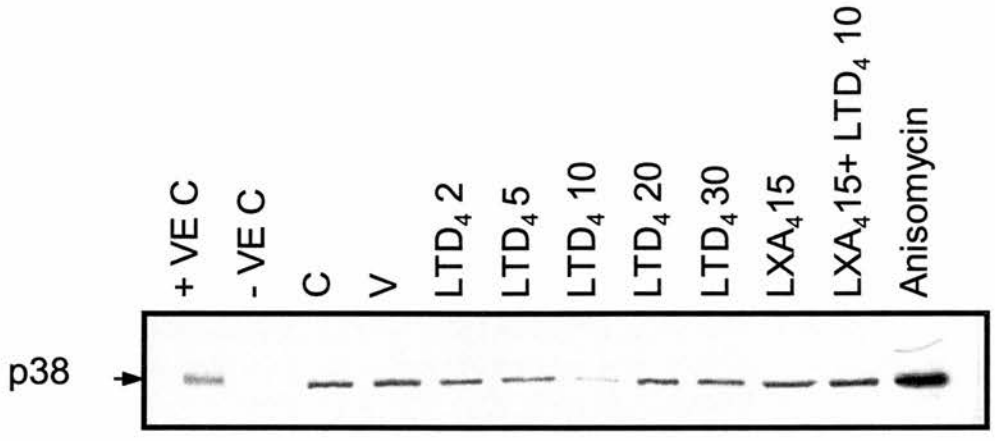


Figure 3.6. p38 activation in ECV-304 cells in response to LTD_4 and LXA_4 stimulation

p38 activation in ECV-304 cells in response to LTD_4 (10^{-7} M) for 2, 5, 10, 20 and 30 minutes. In addition the effects of LXA_4 (10^{-7} M for 15 minutes) either alone or followed by LTD_4 stimulation for 10 minutes was investigated. (lanes 10 and 11). .Total cell extracts from control and anisomycin C-6 glioma cells treated served as positive and negative controls respectively, for phosphorylated and hence activated p38 MAPK (Lanes 1 and 2). Cells treated with anisomycin(10^{-7} M, 15 minutes), a known activator of p38, served as a positive control (Lane 12); vehicle control was ethanol (0.1% v/v). p38 activation occurred under basal conditions and at all conditions and time points studied

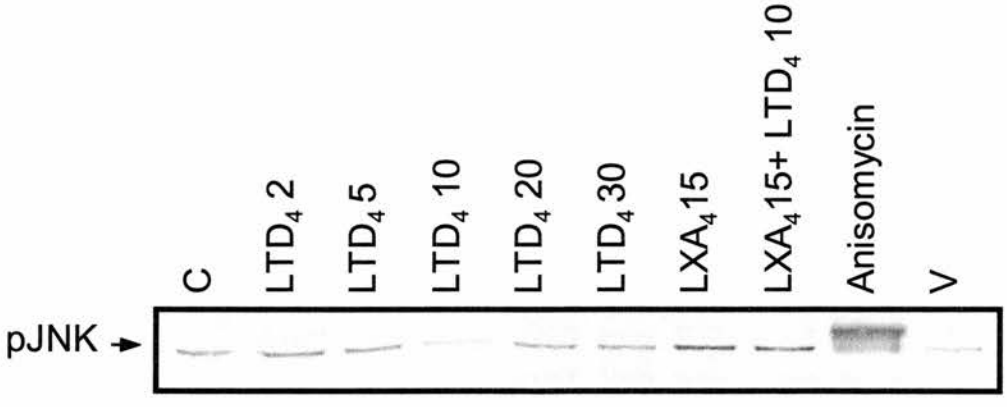


Figure 3.7.JNK activation in ECV-304 cells in response to LTD4 occurred at all time points studied.

Positive (anisomycin) and vehicle controls were included as before. JNK activation occurred at all time points and conditions studied.

Preliminary investigations into the actions of lipoxins on p38 MAPK and JNK activation were incorporated into these experiments. LXA_4 (10^{-7} M, 15 minutes) or LXA_4 (10^{-7} M, 15 minutes) followed by LTD_4 (10^{-7} M 10 minutes) had no apparent effects on kinase activation when compared to controls.

One of the major difficulties in demonstrating increased MAP kinase activity in response to LXA_4 and LTD_4 in ECV-304 cells appeared to be high constitutive levels of kinase activation. Strenuous attempts were made to overcome this, but despite exhaustive efforts, all proved ineffective. Although the original study outlined here had undeniable merits, it became increasingly apparent that continued investigations into lipoxin- and leukotriene-mediated MAP kinase activation in ECV-304 cells were unlikely to yield sufficient data. In addition, further studies into both the upstream mediators linking ligand stimulation to kinase activation and the downstream consequences of this activation in these cells seemed inappropriate. These difficulties also precluded studies using lipoxin analogues in this particular cell line.

The subsequent identification of ECV-304 cells as T24 cells is discussed in section 2.4. No reports can be found concerning the activation status of MAP kinases in T24 cells either constitutively or in response to stimuli. One study however using ECV-304 cells in a model of endothelialisation following denuding injury documented increased expression of ERK, p38 MAPK and JNK following injury (50). The exact reasons for these discrepancies and the findings in the current study are not entirely clear.

Ongoing investigations in our laboratory to establish the signal transduction pathways evoked by lipoxins and leukotrienes in mesangial cells have proved more successful. It appears that in these cells both LXA_4 and LTD_4 activate the MAP kinases (ERK, p38 MAPK and JNK) by a pertussis toxin sensitive pathway (44). This was inhibited by GF109203X, implicating PKC in this process. Only LTD_4-induced MAPK activation was associated with increased activity of phosphoidylinositol-3 (PI-3) kinase, a known upstream activator of ERK, and treatment with a specific LTD_4 receptor antagonist (SKF 104353) blunted both LTD_4-induced MAPK and PI-3 kinase activity. A component of LXA_4-induced ERK activation, however, was insensitive to SKF 104353, pertussis toxin and wortmannin (an inhibitor of PI-3 kinase). It may be, therefore, that LXA_4-induced MAPK activity is mediated by binding at two distinct receptors: one shared with LTD_4 and coupled to a pertussis-toxin sensitive G-protein (G_i) and a second coupled via an alternative G protein such as G_q or G_{12} to ERK activation. In keeping with this hypothesis, evidence has been provided which demonstrates the expression of both the LXA_4 and LTD_4 receptors on mesangial cells.

Recent studies in experimental animal models have confirmed the importance of lipoxins and their stable analogues as potent anti-inflammatory mediators *in vivo* (30, 39, 51). Despite the disappointing results in the current study, given the critical role of endothelial activation in inflammatory events, research should continue to be directed towards defining the molecular mechanisms of lipoxin bioactivity in endothelial cells. ECV-304 cells, used world-wide as an endothelial cell model, were chosen for this study in good faith in an attempt to avoid some of the pitfalls of

primary HUVEC culture. The problems experienced in the current investigation may be avoided by using primary endothelial cells such as HUVEC for further studies.

3.5. Endogenous inhibitors of cytokine bioactivity

Many inflammatory reactions resolve spontaneously suggesting that endogenous "braking systems" exist that "turn-off" leukocyte-mediated inflammation and limit tissue injury. As with the initiation of inflammation, resolution of the inflammatory process occurs via elaboration of a well ordered array of mediators acting at all levels - extracellular, cell surface and intracellular, to down regulate or halt further immune activation and promote recovery. Cytokines, generated from both immune and resident cells, have pleiotropic cellular effects and are central to the generation of the inflammatory response. It is now apparent that, in addition to the generation of certain cytokines which are themselves anti-inflammatory, a number of endogenous inhibitors of cytokine bioactivity exist which are important in inflammatory resolution. For the purposes of this discussion glomerulonephritis (GN) will be viewed as a paradigm of pathogenic inflammation and reference made to *in vivo* and *in vitro* studies which have attempted to characterise the mechanisms which limit such immune-mediated damage within the glomerulus.

The different varieties of GN may be distinguished on the basis of their initiating stimuli, histological features and the nature of their progression, but in all forms the normal, and usually tightly regulated, immune response undergoes inappropriate activation. In the most simplistic of terms the process may be divided into four stages: *initiation* (immune complex formation and antigenic-stimulation of T cells), *leukocyte recruitment* (the extravasation of circulating monocytes/macrophages from the blood vessel lumen into the glomeruli and/or tubulointerstitum), *proliferation* (predominantly of resident mesangial cells) and and either *scarring* (as a result of extracellular matrix accumulation) or *resolution*. Throughout the inflammatory

process is sustained and amplified via the release of cytokines and monocyte/T cell chemotactic factors by both activated resident cells and infiltrating immune cells.

3.5.1. Anti-inflammmatory cytokines

The cytokines interleukin (IL)-4, IL-10 and IL-13 are produced mainly by T helper type 2 (Th 2) cells and exert broadly anti-inflammatory effects (reviewed in (3) and references contained therein). These so-called Th2 cytokines have multifunctional roles, the most important of which is the modulation of macrophage function. Th 2 cytokines have been documented to inhibit macrophage release of reactive oxygen species (ROS), proinflammatory cytokines and chemokines. In addition, these anti-inflammatory mediators down-regulate leukocyte growth factors (granulocyte macrophage colony-stimulating factor (GM-CSF) and granulocyte colony stimulating stimulating factor (G-CSF). All of these effects result in a marked attenuation of the inflammatory process. Administration of IL-4, for example, in a murine model of crescentic glomerulonephritis induced by anti-glomerular basement membrane (anti-GBM) antibody, has been shown to reduce crescent formation, attenuate macrophage/T cell infiltration and decrease proteinuria. Conversely deletion of the IL-4 gene in mice by homologous recombination (i.e. "knock-out" technology) exacerbates nephritis. In addition to its effects on macrophages, IL-4 can inhibit release of ROS from neutrophils and modulate the function of resident cells e.g. inhibition of IL-1-induced proliferation of mesangial cells. IL-10 has also been shown to have immuno-suppressive effects in the anti-GBM model of GN. Renal expression of IL-13 has also been demonstrated in experimental models of glomerulonephritis, although the putative anti-inflammatory role of this cytokine *in vivo* in the resolution of kidney disease has yet to be clarified. Interestingly, in a

murine model of collagen-induced arthritis, treatment with vector cells engineered to secrete IL-13 resulted in attenuation of inflammation.

IL-6 is produced in almost all inflammatory states, often in conjunction with the pro-inflammatory cytokines TNF-α and IL-1. IL-6 is critical to the generation of immunity against chronic intracellular infections, and, together with TNF-α and IL-1, is required for the induction of acute phase reactions (fever, corticosterone release and hepatic production of acute phase proteins). Up until recently it was unclear whether IL-6 made any additional contribution to acute inflammatory responses, and indeed conflicting reports existed as to its pro- or anti-inflammatory effects. IL-6 for example has been shown to induce proliferation of mesangial cells *in vitro* and *in vivo* and administration of IL-6 exacerbates murine lupus nephritis, while blockade of the IL-6 receptor promotes disease resolution (3). Subsequent investigations however failed to demonstrate IL-6 induction of mesangial cell proliferation and in nephrotoxic serum nephritis IL-6 administration has been reported to reduce glomerular thrombosis, leukocyte infiltration, expression of cytokines/chemokines and proteinuria. A recent study investigating the role of IL-6 in animal models of endotoxic lung/endotoxaemia in IL-$6^{+/+}$ and IL-$6^{-/-}$ mice has helped to clarify its role in acute inflammation (52). Aerosol exposure of mice to endotoxin induced a release of TNF-α and MIP-2, in addition to a neutrophil response, in the lungs of IL-$6^{+/+}$ mice. These effects however were significantly greater in the lungs of IL-$6^{-/-}$ mice. Systemic responses following intraperitoneal delivery of endotoxin were also more pronounced in the IL-$6^{-/-}$ mice, as demonstrated by increased circulating levels of TNF-α, MIP-2, GM-CSF and IFN-γ. Administration of recombinant IL-6 to IL-$6^{-/-}$ mice abolished these differences. Importantly, the anti-inflammatory cytokine IL-10

was induced to a similar degree in both sets of mice following endotoxin challenge. This provides strong evidence that endogenous IL-6 plays an important anti-inflammatory role in both local and systemic acute inflammatory responses by controlling the level of pro-inflammatory cytokines.

Leukaemia inhibitory factor (LIF), an IL-6 family member, is another pleiotropic cytokine produced by many different cell types including endothelial and mesangial cells. Low levels are expressed in normal rat glomeruli but increase rapidly and dramatically in response to induction of nephritis. Administration of LIF in experimental anti-GBM nephritis results in amelioration of disease with reduced macrophage infiltration and decreases in MCP-1, IL-1 and TNF-α mRNA expression.

The complex actions of the cytokine TGF-β are essential in a wide range of pathophysiological processes (3). Constitutive expression is essential for the maintenance of normal immune and tissue function; TGF-β "knock-out" mice demonstrate immune dysregulation, multiorgan inflammation and early death (53). TGF-β is upregulated in many diseases particularly autoimmune disorders where it exerts anti-inflammatory actions. TGF-β1 is upregulated in many forms of experimental and human glomeronephritis (54). Production appears to be mainly from the mesangial cell and can be enhanced *in vitro* by diverse stimuli such as angiotensin II, thrombin, high glucose, oxidised LDL, immune complexes and mechanical strain. *In vitro* studies have also demonstrated that, in addition to functioning as an autocrine inhibitor of the cytokine response in mesangial cells (IL-

1β and TNF-α), TGF-β1 production from these cells inhibits production of IL-1α, TNF-α, interferon (IFN)-γ and MCP-1 by activated macrophages. This has been borne out by studies using an *ex vivo* gene transfer approach for TGF-β1 delivery where a sustained increase in TGF-β1 glomerular expression was associated with a decreased glomerular cell proliferation in experimental mesangial proliferative glomeruonephritis. TGF-β however is a double-edged sword in that, in addition to its immunosuppressive actions, this cytokine also exerts potent fibrogenic effects. Introduction of the TGF-β into the kidney induces glomeruloscelerosis *in vivo* (55), while *in vitro* studies have confirmed increased mesangial cell synthesis of collagen, fibronectin and proteoglycans in response to TGF-β, concomitant with a decreased catabolism of these compounds. Indeed it has become accepted that the accumulation of extracellular matrix, the characteristic hallmark of glomerulosclerosis, is predominantly due to the pro-fibrogenic/anti-proteolytic consequences of excess TGF-β production in renal inflammation.

3.5.2. Inhibition of cytokine bioactivity by anti-inflammatory eicosanoids

The activation of monocytes/macrophages and polymorphonuclear cells (PMN) in inflammatory states is accompanied by production of a number of arachidonic acid derived metabolites which have important immunomodulatory effects (56). In the majority of cases these are pro-inflammatory and include thromboxane A_2, prostaglandin (PG) $F_{2\alpha}$, leukotrienes B_4, C_4 and D_4. These mediators trigger leukocyte infiltration, coagulation, and proliferation/matrix production by resident cells. In some cases however eicosanoids function as anti-inflammatory agents and these include prostacyclin (PGE_2, PGI_2) and lipoxins (LX). The latter compounds

exert a number of anti-inflammatory actions (section 3.2). With particular reference to cytokine bioactivity, PGE_2 and PGI_2 inhibit production of IL-1β and TNF-α from activated macrophages. Production of PGE_2 *in vivo* occurs in many inflammatory states, while *in vitro* studies e.g. with mesangial cells, have shown increased production in response to a variety of inflammatory stimuli (IL-1β, TNF-α, ROS, angiotensin II, thrombin, immunoglobulins).

3.5.3. Cytokine inhibitors: antagonists

The ability to elaborate a set of natural inhibitors against specific cytokines is another important contributor to the resolution of established inflammation. IL-1 receptor antagonist (IL-1ra) is one such mediator (56). IL-1ra (which is homologous to IL-1α and IL1β) competes with IL-1 for binding at its receptors but, in contrast to IL-1, fails to induce interiorisation of receptor-ligand complexes and stimulate intracellular responses. This antagonism by IL-1ra results in the attenuation of the potent pro-inflammatory effects of IL-1. The expression of IL-1ra has been demonstrated in many inflammatory diseases and attempts have made to utilise its anti-inflammatory effects by upregulating its expression either by administration of recombinant IL-1ra protein or by gene transfer techniques. Injection of IL-1ra limits glomerulonephritis in a number of experimental models at various stages of the disease process, while transfer of mesangial cells expressing IL-1ra to glomeruli results in their insensitivity to IL-1. *In vitro* studies also indicate that IL-1ra expression in macrophages is upregulated not only by IL-1, but also by the anti-inflammatory cytokines IL-4, IL-6 and TGF-β.

Decorin is a proteoglycan that binds TGF-β isoforms and neutralises their activity (3). In acute anti-Thy 1 glomerulonephritis systemic administration of decorin, or indeed intramuscular transfer of its gene, results in inhibition of matrix expansion and proteinuria. No information exists on whether decorin inhibits the positive immunosuppressive effects of TGF-β.

3.5.4. Cytokine inhibitors:soluble receptors/decoy receptors

Bioactivity of the pro-inflammatory cytokines IL-1 and TNF-α may also be modulated by naturally occuring soluble binding proteins (56). In the case of IL-1 two receptors, type I and type II, (RI and RII) are co-expressed in different cell types. IL-1 signalling occurs principally via RI while RII has no signalling properties and acts as a "decoy" for IL-1. This results in an inhibition of IL-1 activity by preventing its binding at the active R1 receptor. In addition, activated inflammatory cells secrete a soluble form of both receptors that retain the ability to bind IL-1 and, hence, compete with its membrane receptors for binding. The efficacy of these molecules as potential therapeutic agents has been assessed in experimental glomerulonephritis where their administration limits glomerular injury.

Soluble TNF receptors (sTNFRp55 and sTNFRp75) have also been identified (56). These are truncated forms of membrane TNF-α receptors which appear to function as antagonists by competitively inhibiting TNF interaction with cell-surface receptors. *In vivo* recombinant analogues of the soluble TNF receptors show potent TNF-neutralising activity e.g. in the rat model of acute nephrotoxic serum nephritis administration results in blunting of the inflammatory response with reduced expression of glomerular adhesion molecules and decreased leukocyte infiltration.

Subsequent to these changes, a suppression of histopathological changes (intracapillary thrombi and crescent formation) and preservation of renal function. The presence of soluble TNF receptors has been identified in the urine of patients with chronic glomerular nephritis and it has been speculated that these may be produced in response to inflammatory stimuli in the glomerulus to limit the action of TNF-α.

3.5.5. Intracellular inhibitors of cytokine cell signalling events

Cytokines initiate their pleiotropic cellular effects through their interactions with specific receptors on the surface of target cells. Once bound to the surface receptor various intracellular signal transduction pathways are activated and these lead ultimately to the transcription of specific genes. Cytokine signalling from receptor to nucleus occurs through three principal pathways - the Janus kinase / signal transducers and activators of transcription (JAK/STAT) cascade, mitogen-activated protein kinases and NF-κB activation. In the JAK/STAT pathway binding of cytokine to receptor results in the juxtaposition and activation (presumably by transphosphorylation) of the receptor associated JAKS (57). The activated JAKS phosphorylate a number of key tyrosine residues in the cytoplasmic portion of the cytokine receptor subunits. These phosphotyrosines are then believed to act as docking sites for the src homology 2 (SH2) domains of STAT transcription factors. The recruited STATs are activated, via JAK mediated phosphorylation, and they then dislocate from the receptor complex and form homo- or heterodimers. Finally, the STAT dimers translocate to the nucleus where they bind to specific DNA elements in gene promoters and thus induce transcription of cytokine dependent genes (Fig 3.8). Recently a family of inhibitors of JAK/STAT signalling have been reported, the

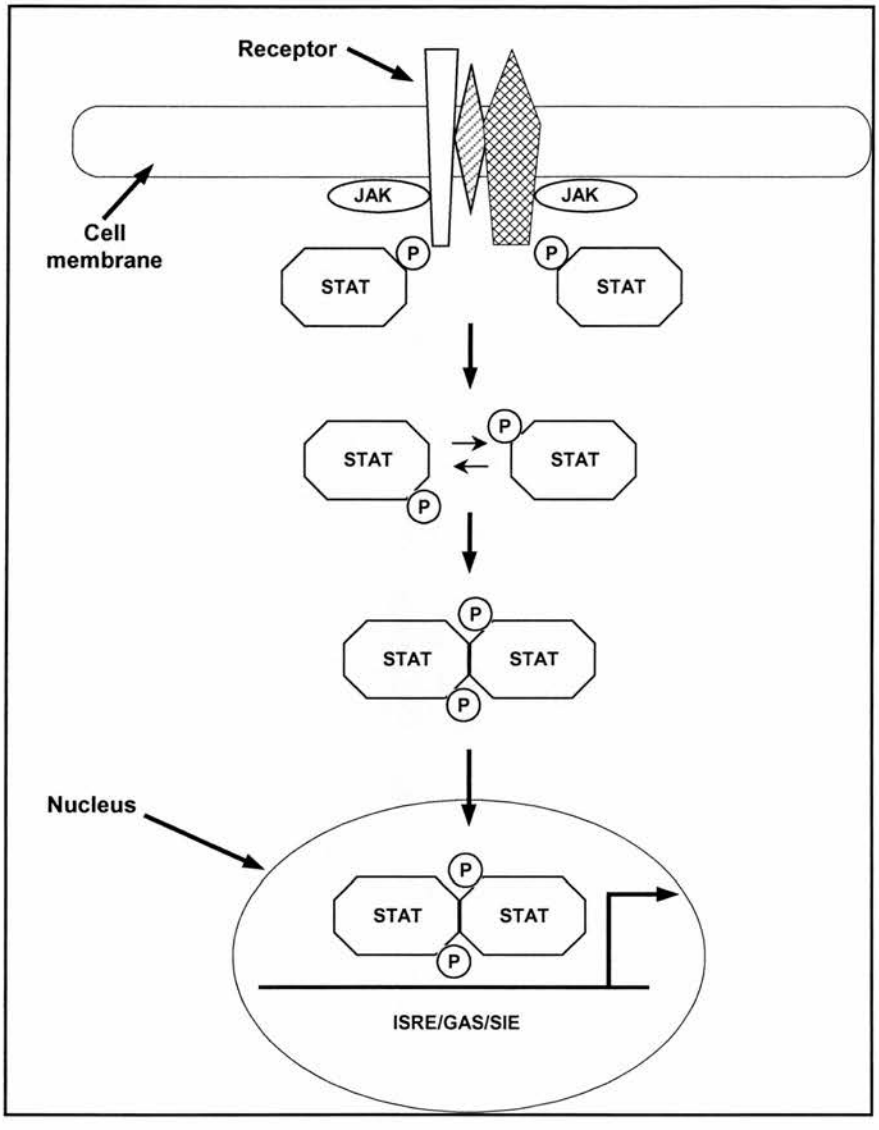


Figure 3.8. Cartoon summarising the key elements of the JAK/STAT pathway (see text for details).

suppressors of cytokine signalling (SOCS) family (58-61). The SOCS proteins are products of JAK/STAT-inducible early genes and function, via a classic negative feedback loop, to down regulate this pathway. In essence, cytokine-mediated activation of the JAK/STAT pathway leads to the transcription of various genes, including those encoding SOCS proteins; thus, by activating the JAK/STAT cascade,

cytokines are also initiating the process by which they will be eventually 'switched off'. The SOCS proteins will be discussed in detail in section 3.6.

In addition to SOCS, another family of proteins have been identified which inhibit STAT mediated gene transcription – protein inhibitor of activated STAT (PIAS) proteins (62). These proteins exist constitutively in the cytoplasm of cells and upon cytokine binding preferentially associate with phosphorylated STATS. In the case of PIAS 1 and PIAS 3, their association with STAT 1 and STAT 3, respectively, inhibits STAT-mediated gene activation, most likely by blocking their DNA-binding ability. It has been suggested that the striking specific *in vivo* association between individual PIAS and STAT proteins may indicate the presence of a specific PIAS inhibitor in every STAT signalling pathway, but this remains to be clarified.

Activation of the various MAPKs results not only in their phosphorylation of cytoplasmic proteins, but also in their translocation to the nucleus where phosphorylation can activate various transcription factors (section 3.3). Phosphorylation in these systems is a dynamic process balanced by the actions of the kinases which catalyse phosphorylation and the phosphatases which catalyse dephosphorylation. MAP kinase phosphatase (MKP)-1 is one such phosphatase. MKP-1 suppresses the activity of the MAPK family and has recently been shown to be upregulated in the glomeruli of anti-Thy 1 glomerulonephritis (3). In addition, MKP-1 has been implicated in the very early stages of anti-GBM GN. Injection of anti-GBM antibodies to rats resulted in an increase in MKP-1 levels as early as 30 min, that returned to basal levels after 24 hours. The significance of these findings is still unclear.

The transcription factor NF-κB resides normally in the cytoplasm of resting cells, but enters the nucleus in response to various stimuli such as infection, ultraviolet irradiation and pro-inflammatory cytokines such as TNF-α and IL-1β. In the cytosol NF-κB is complexed with its inhibitor IκB and its activation is controlled by inhibitory IκB kinases (IKK) (63). Signal-induced phosphorylation of IκB by IKK initiates its conjugation to ubiquitin and subsequent degradation by the proteasome. The liberated NF-κB can then translocate to the nucleus and activate gene expression (Fig. 3.9).

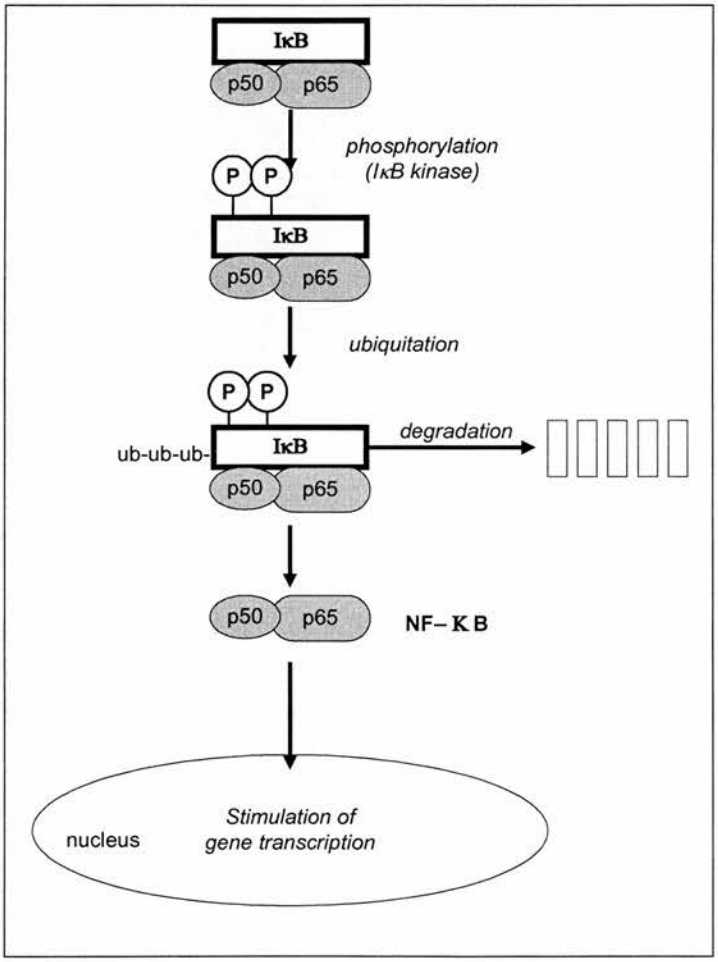


Figure 3.9. NF-κB activation.

The activity of IKK resides in two subunits IKKα and IKKβ which share significant homology and contain identical structural domains, but mediate quite different NF-κβ dependent effects. In recent studies $IKK\alpha^{-/-}$ mice died shortly after birth and demonstrated striking limb and skeletal abnormalities (64). The mediators controlling IKKα activity early in development are unknown, but are unlikely to be IL-1β or TNF-α because both these cytokines can activate NF-κβ in cells from the $IKK\alpha^{-/-}$ mice. IKKβ on the other hand appears to control the NF-kβ responsive genes involved in the inflammatory response e.g. TNF-α and IL-1β. It has been shown that inhibition of TNF-α induced NF-κβ activation by salicylate (the hydrolysis product of aspirin) occurs via suppression of IKKβ activity. Cells lacking NF-κB activity have also been shown to undergo apoptosis in response to TNF-α and, in support of IKKβ control of TNF-α induced effects, $IKK\beta^{-/-}$ mice died in utero of TNF-α-induced hepatocyte apoptosis (65). Because of the potential toxicity and pathophysiology associated with prolonged NF-κB activation, it is important not only to activate this system rapidly in response to infection, but also to decrease its activity once the infectious challenge disappears. The mechanisms involved in down regulation of this pathway are still unknown but it has been suggested that IKKβ may be a site for negative regulation of IKK, and hence, NK-κB activity (66). IKK-β is rapidly activated through phosphorylation at its activation (T) loop and also at a COOH-terminal serine cluster. The region encompassing the serine cluster appears to be an intrinsic activator of the kinase and it has been speculated that the - COOH-terminal portion of the molecule folds back to contact the kinase domain and induces a conformational change that enhances catalytic activity. Progressive

phosphorylation of the serine cluster may weaken these interactions and act as a timing device that limits the period of IKK activation.

3.5.6. The acquisition of an anti-inflammatory phenotype

In addition to anti-inflammatory cytokines or inhibitors of proinflammatory cytokines, a large number of other mediators exist which result in down-reguation of the immune response in other ways These include complement inhibitors, anti-thrombotic molecules, heat shock proteins, antioxidant enzymes and cyclin kinase inhibitors and are discussed in detail elsewhere (3). Many of these so-called "defence" molecules are constitutively expressed in normal tissues and may be involved in the prevention/limitation of injury and the maintenance of tissue integrity. Others are inducible in response to a local inflammatory milieu and contribute to the resolution of inflammation. It is interesting that one defending molecule may trigger the expression of another or act in synergism with it against the inflammatory stimulus. IL-1 and TGF-β for example trigger the expression of their specific inhibitors IL-1ra and decorin. TGF-β induces IL-10, while IL-10 itself upregulates IL-1ra and sTNFR. IL-4 and IL-13 stimulate expression of IL-1ra, IL-1 decoy receptor and 15-lipoxygenase (the enzyme responsible for lipoxin formation). The acquisition of this so-called "anti-inflammatory phenotype" has been supported by *ex vivo* data. In the regeneration phase (day 7) of acute anti-thy 1 glomerulonephritis, glomeruli have been reported to show blunted responses to pro-inflammatory stimuli (67). When normal glomeruli were stimulated with IL-1β the expression of the cytokine-responsive matrix metalloproteinase stromelysin was induced. This expression was blunted however in nephritic glomeruli, which also showed reduced expression of other cytokine inducible molecules including

gelatinase B and MCP-1. In nephritis blunted glomerular responses were also observed in response to lipopolysaccaride and TPA. In the light of these findings, it has been speculated that the elaboration of defending molecules such as those detailed above in previous sections contributes to this reduced responsiveness, leading the authors to hypothesise that such desensitisation of resident tissue represents an "anti-inflammatory phenotype" which both limits cell damage and facilitates disease resolution.

3.6. Suppressors of cytokine signalling (SOCS) proteins

Cytokines are secreted proteins which regulate the proliferation, differentiation and activation state of many different cell-types. They are important modulators of diverse homeostatic processes such as development, haematopoiesis and host defence. Inappropriate cytokine release contributes to tissue injury and organ dysfunction in patients with autoimmune disease. Cytokines initiate their pleiotropic effects through interaction with specific cell surface receptors on target cells. Receptor engagement activates intracellular signal transduction pathways that evoke diverse functional responses, including transcription of new genes. A recently identified family of proteins, the suppressors of cytokine signalling (SOCS), which act as negative regulators of a key cytokine-activated signalling pathway, the Janus kinase/*s*ignal *t*ransducers and *a*ctivators of *t*ranscription (JAK/STAT) cascade, appear to be important regulators of cytokine bioactivity in health and disease (58-61).

3.6.1. The JAK/STAT pathway

Over the past decade the importance of the JAK/STAT signal transduction cascade in linking activation of cytokine receptors to gene transcription has been established (57). In unstimulated cells JAKS are associated with cytokine receptors. Following cytokine binding, receptor aggregation results in the juxtaposition and activation (presumably by transphosphorylation) of the associated JAKS. The activated JAKS phosphorylate a number of key tyrosine residues in the cytoplasmic portion of the receptor subunits. These phosphotyrosines are then believed to act as docking sites for the src homology 2 (SH2) domains of STAT transcription factors. The recruited STATs are activated via JAK mediated phosphorylation of a phosphotyrosine residue

carboxyl to the SH2 domain (Tyr-701 in the case of STAT 2) and they then dislocate from the receptor complex and form homo- or heterodimers via reciprocal interactions between their SH2 domains and the phosphotyrosine residue. Finally, the STAT dimers translocate to the nucleus where they bind to specific DNA elements in gene promoters and thus induce transcription of cytokine dependent genes. Most definitive studies on JAK/STAT activation have been carried out using with interferon (α and γ) and have identified two major prototypic sequences in the genes upregulated by these cytokines – the interferon α stimulated response element (ISRE) and the interferon γ inducible element, or GAS element (68).

3.6.2. SOCS: discovery, structural features and regulation of expression

Whereas the key events in cytokine-mediated JAK/STAT activation have been relatively well-defined (Fig 3.10), until recently little was known of the mechanisms of inactivation of this signal transduction pathway.

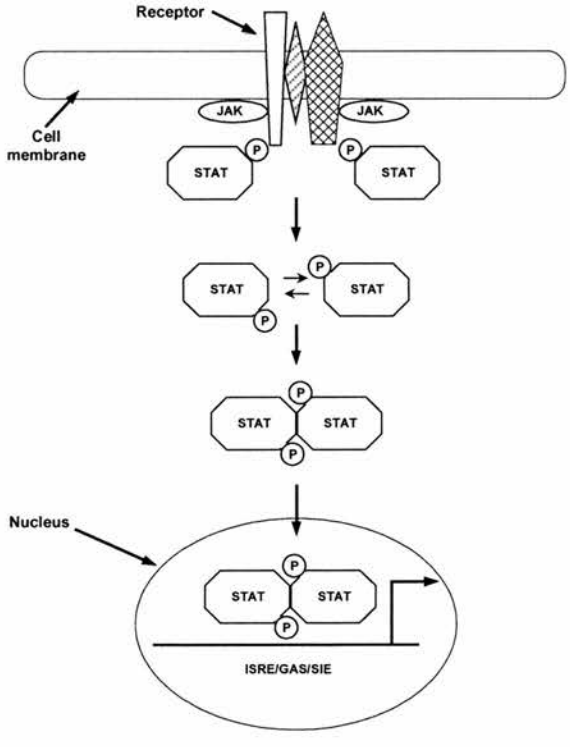


Figure 3.10. JAK/STAT pathway.

Over the past four years a family of inhibitors of JAK/STAT signalling has been characterised, the *s*uppressors *o*f *c*ytokine *s*ignalling (SOCS) family – alternatively termed *c*ytokine *i*nducible SH2-containing *s*equence, CIS/ STAT-induced STAT inhibitor, SSI / JAK binding protein,

JAB (58-61). The SOCS proteins are products of JAK/STAT-inducible immediate early genes and function as negative regulators of this pathway (Fig 3.11).

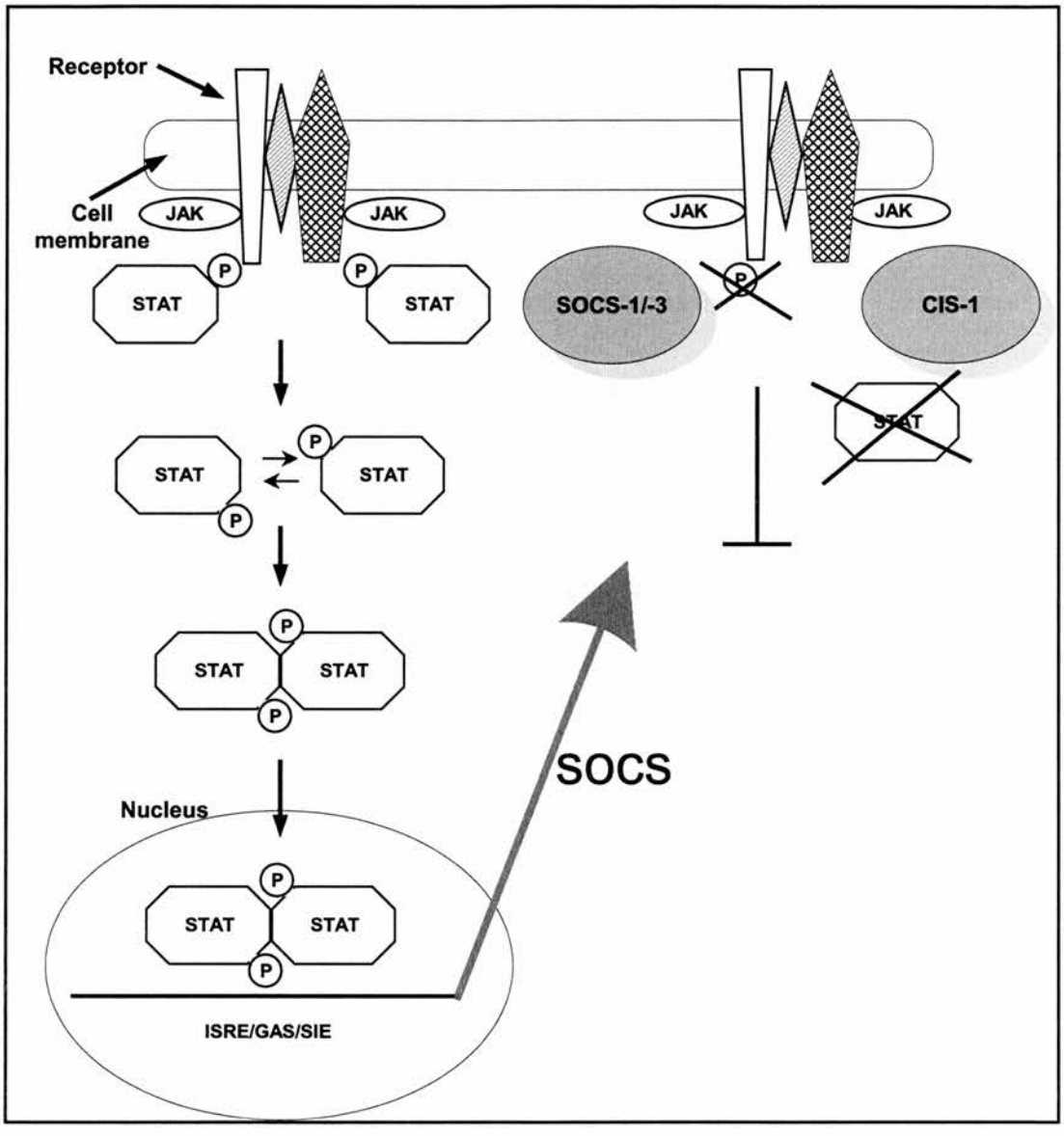


Figure 3.11. SOCS proteins

The SOCS proteins are products of JAK/STAT-inducible early genes and function, via a classic negative feedback loop, to down regulate this pathway. In essence, cytokine-mediated activation of the JAK/STAT pathway leads to the transcription of various genes, including those encoding SOCS proteins; thus, by activating the JAK/STAT cascade, cytokines are also initiating the process by which they will be eventually 'switched off'

To-date the SOCS family comprise at least eight members, SOCS-1–7 and CIS-1. CIS-1, the first member identified, is the product of a cytokine-inducible gene and binds to the phosphorylated IL-3 and erythropoietin (Epo) receptors (61). Epo, while historically classified as a hormone, is a member of the growth hormone family of cytokines and the Epo receptor a member of the class 1 cytokine receptor family (57). SOCS-1 was subsequently identified almost simultaneously by four independent groups using a variety of experimental approaches, a discovery that was quickly followed by the identification of multiple murine and human SOCS members (69, 70).

Basal and cytokine induced SOCS expression exhibits striking tissue- and cell-type specificity; for example, interleukin (IL)-6 is capable of upregulating the expression of SOCS-1, -2 and -3 in mouse liver, while this cytokine induces only SOCS-1 in murine monocytic M1 cells (60). Similarly, although CIS-1 induction was originally thought to be IL-6 independent (61), it has since been shown to be upregulated in a cell- and tissue-type specific manner in response to this cytokine.

SOCS over-expression and promoter analysis studies support the theory that cytokine-mediated SOCS upregulation is due to STAT-dependent transcription. A dominant–negative STAT 3 mutant has been shown to block IL-6 and leukaemia inhibitory factor (LIF)-dependent SOCS-1 upregulation (71). The rat SOCS-1 promoter contains several GAS consensus motifs (72). Four STAT 5 consensus binding sites located on the CIS-1 promoter close to the transcription initiation site are also required for Epo-dependent upregulation of this gene (73) and characterisation of the murine SOCS-3 promoter has revealed a STAT-1/STAT-3

binding element essential for LIF-dependent transcription (74). Whereas cytokines appear to be the principal mediators of JAK/STAT-driven SOCS upregulation, other ligands capable of initiating STAT transcriptional activation e.g. epidermal growth factor and platelet derived growth factor, are also potential inducers of these negative regulators. Interestingly, there is evidence that SOCS-3 is upregulated by IL-10 via a STAT-independent mechanism, implying that additional cytokine-activated transcription factors are involved in SOCS upregulation (75). Moreover, the recent intriguing observation that SOCS-2 is physically associated with the activated insulin-like growth factor-1 receptor (76) has led to speculation that the role of SOCS as regulators of intracellular signalling may extend beyond their inhibition of JAK/STAT activity.

3.6.3. Structural elements

The most striking structural feature of the SOCS family members is a novel conserved C-terminal motif of approximately 40 amino acids containing a core conserved sequence - (K/R)(D/E)(Y/F). This has been alternatively termed the SOCS box, the CIS homology (CH) domain or the SSI C-terminal motif (SC motif). The SOCS box is conserved among family members both at the level of its amino acid sequence and its position within individual SOCS molecules, suggesting that this motif may serve a similar function in each protein (77). SOCS-1-7 and CIS-1 have a Src homlogy 2 (SH2) domain amino terminal to the SOCS box. The latter observation has prompted speculation that these members of the SOCS family may bind to phosphorylated tyrosine residues on target proteins via this region. Indeed, SOCS-1 has been reported to bind to the kinase domain of JAK2 as a result of SH2:phosphotyrosine interactions (58). A further 8 proteins containing the SOCS

box and featuring additional amino terminal protein-protein interaction motifs (specifically WD-40/ankyrin repeats and SPRY domains) have also been identified (77). In addition, two small GTPases and two small sequences of unknown structural class which contain a C-terminal SOCS box have also been reported.

Contrary to initial predictions the SOCS box does not appear to be required for inhibition of cytokine signalling (78-80). Evidence has been provided that the SOCS box may be involved in *preventing* the proteolytic breakdown of SOCS proteins in some systems; whereas the SOCS box may be responsible for *targeting* SOCS proteins for proteosomal degradation in others (81). In support of the former, SOCS-1 mutants lacking the SOCS box are expressed at lower levels in cultured cells than the full length protein; a phenomenon that is reversible in the presence of proteasome inhibitors (79). Elongins B and C are proteases that putatively target proteins to proteasomal destruction and SOCS proteins appear to interact with the Elongin BC complex via a BC interaction motif located within the SOCS box. In one study Elongin BC-SOCS interaction stabilises SOCS-1 levels by rendering the molecule relatively resistant to proteolytic degradation (78). In contrast, Zhang et al. have reported that proteasome inhibition prolongs cytokine-induced SOCS-3 upregulation and suggested that the proteasomal complex, and by inference the Elongin BC proteins, may facilitate the degradation of endogenous SOCS proteins (81). Clearly, further research in this area is required to clarify these apparently contradictory findings.

3.6.4. Mechanisms of inhibition of JAK/STAT signalling

SOCS have been reported to inhibit cytokine-triggered JAK/STAT signal transduction pathways through several mechanisms. SOCS-1 appears to associate directly with JAKS and thereby inhibit kinase activity and STAT-dependant gene transcription. In contrast, CIS-1 does not appear to physically interact with JAK-2 but inhibits binding of STAT-5 to cytokine receptors (73). Furthermore, SOCS-3 interacts with JAK-2 *in vitro* (58) but suppresses JAK activity weakly (82, 83) even though it inhibits cytokine bioactivity. In the case of SOCS-1 and SOCS-3, intact SH2 and N-terminal sequence are also required for inhibition of IL-6 signalling (79, 80), further highlighting the complexity of this system.

SOCS-1 has also been demonstrated to inhibit cytokine-dependent activation of the non-receptor tyrosine kinase Tec (82), although the role of this kinase in JAK/STAT signalling remains to be clarified. In the latter study, SOCS-1 over-expression suppressed both Tec kinase and JAK-2 activity in haematopoietic cells; however the suppression of JAK-2 was more consistent. These observations led the authors to speculate that SOCS-1 has a higher binding affinity for JAK-2 than Tec, and that JAK-2 was the more likely target of SOCS-1.

3.6.5. Physiological roles

Complementary experimental approaches using (a) analyses of SOCS expression under basal conditions and following cytokine stimulation, (b) SOCS over-expression systems and, (c) SOCS 'knockout' mice, have begun to yield valuable insights into the function of these proteins in health and disease. Most studies point to SOCS as important negative regulators of JAK/STAT signalling triggered by

hormones and cytokines. Growth hormone (GH), for example, produces a rapid transient induction of SOCS- 3 in fibroblasts *in vitro* (84). Intraperitoneal administration of GH to mice *in vivo* causes induction of SOCS-3 in the liver. Transfection of chinese hamster ovary (CHO) cells with SOCS-1 and -3 blocked GH-induced transactivation of a GH responsive gene promoter, evidence which strongly implicates SOCS-1 and 3 as inhibitors of GH-JAK 2 signalling (84).

SOCS-3 also regulates leptin signalling *in vivo* (85). Leptin administration to leptin-deficient mice rapidly induces SOCS-3 mRNA in the hypothalamus and over-expression of SOCS-3 blocks leptin receptor-mediated signal transduction in COS-1 cells (86). Furthermore, in the lethal yellow mouse (A^Y/a), a model characterised by hyperleptinaemia and resistance to both central and peripheral leptin administration (86), basal SOCS-3 mRNA are increased in hypothalamic nuclei. It has been speculated that excessive SOCS-3 activity in leptin resistant neurons might be a potential mechanism for this pathology in the A^y /a mouse, and indeed other syndromes of obesity. SOCS-3 is also a negative regulator of leukaemia inhibitory factor (LIF)-induced proopiomelanocortin gene expression and subsequent ACTH secretion *in vitro* and *in vivo* (87).

The growth, differentiation and functions of immune and haematopoietic cells are controlled by multiple cytokines. The importance of SOCS in modulating the effects of cytokines on haematopoietic cell lines has been the subject of intense study. SOCS-1 is strongly induced by IFN-γ in mouse myeloid leukaemia (M1) cells (88). Over-expression of SOCS-1 in these cell lines causes resistance to interferon-induced growth arrest. It has been postulated that this may be a contributory mechanism

underlying IFN resistance, for example in the late phase of chronic myeloid leukaemia (89). The expression of CIS-1 mRNA and protein has also been demonstrated in haematopoietic cell lines. CIS-1 protein interacts with the IL-3 receptor and with the tyrosine phosphorylated erythropoietin receptor. The over-expression of CIS-1 in various haematopoietic cell lines suppresses cell growth (61) implicating CIS-1 protein as a negative regulator of cytokine growth signals.

Mice deficient in SOCS-1 protein exhibit stunted growth and abnormalities in a range of organs (71, 90). Although normal at birth, the mice die before three weeks of age. Histological examination of the liver revealed fatty degeneration of parenchymal cells, as well as generalised infiltration of the liver by immature haematopoietic cells. These changes were also present, though less marked, in pancreas, lungs and heart. Conversely, the lymphoid organs, thymus and spleen, showed markedly reduced cellularity with progressive depletion of pre-B and B cells. Indeed, while the differentiation of lymphocytes lacking SOCS-1 appeared to be normal, these cells exhibited accelerated apoptosis with ageing, the number of lymphocytes in SOCS-1 homozygous deficient mice at 10 days being 20-25% of their wild type counterparts. These authors found that expression of the pro-apoptotic protein Bax was elevated in spleen- and thymus-derived lymphocytes of SOCS-1 deficient mice, leading them to speculate that SOCS-1 might function to prevent lymphocyte apoptosis by inhibiting the expression of this molecule.

3.6.6. Summary

In summary, cytokine-triggered phosphorylation of STATs induces the transcription of SOCS genes. The SOCS proteins, in turn, inhibit cytokine signal transduction and

represent a classical negative feedback loop. They are also ideally placed to participate in crosstalk among cytokine signals because the SOCS genes induced by one cytokine could potentially modify the intensity of other cytokine signals. This paradigm has been suggested as a possible mechanism underlying the inhibition of IFN-dependent gene induction by IL-10 (91) and a recent publication has provided evidence that IFN-dependent SOCS-1 upregulation may be, at least partially, responsible for the inhibitory effect of this cytokine on IL-4-dependent gene expression (92).

3.7. Regulation of SOCS expression in glomerular endothelial and mesangial cells *in vitro* and *in vivo*

3.7.1. Introduction

Glomerulonephritis (GN) can be viewed as a paradigm of autoimmune inflammation in which the normal, and usually tightly regulated, immune response undergoes inappropriate activation. In the most simplistic of terms the process may be divided into four stages: *initiation* (immune complex formation and antigenic-stimulation of T cells), *leukocyte recruitment* (the extravasation of circulating monocytes/macrophages from the blood vessel lumen into the glomeruli and/or tubulointerstitum), *proliferation* (predominantly of resident mesangial cells) and either *scarring* (as a result of extracellular matrix accumulation) or resolution. Throughout the inflammatory process is sustained and amplified via the release of cytokines and monocyte/T cell chemotactic factors by both activated resident cells and infiltrating immune cells. Considerable efforts have been made to characterise the mechanisms which limit such immune-mediated damage within the glomerulus and these are discussed in more detail in section 3.5. Since the role of cytokines in the pathology of GN is well established (93), and the potent inhibition of JAK/STAT signaling by SOCS establishes their importance as endogenous regulators of key cytokine-mediated biological processes, the present study was undertaken to elucidate the role of SOCS in renal inflammation.

3.7.2. Materials and methods

Cell Culture

Transformed rat glomerular endothelial cells were a generous gift from Dr. Harry Holthofer and have been characterised previously (94). All cell culture reagents were obtained from Gibco Life Technologies (Paisley, Scotland) unless other wise stated. These cells were routinely cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing L-alanyl-L-glutamine, sodium pyruvate, 1000 mg/L glucose and pyridoxine and supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Prior to stimulation RGEC were not rendered quiescent, as they were found to detach under conditions of serum restriction. Cytokines were obtained from R&D systems (Oxford, UK) and all treatments were carried out at a concentration of 100 ng/ml for 2 hours.

Primary cultures of rat mesangial cells were isolated as described previously (95) and were a kind gift of Dr Ann M^{c}Ginty. Cells were cultured in RPMI ((Biowhittaker, Wokingham, UK), 17% heat-inactivated fetal bovine serum 100 U/ml penicillan and 100 μg/ml streptomycin. Cells were plated onto 6-well plates and when 80% confluent rendered quiescent overnight in RPMI, containing 0.5% fetal bovine serum. Cytokines were obtained from R&D systems (Oxford, UK) and all treatments were carried out at a concentration of 100 ng/ml for 2 hours.

Rat Crescentic Nephritis Model

Crescentic GN was induced in male Wistar Kyoto (WKY, 200-250 g) rats by a single injection (0.1 ml) of rabbit anti-rat GBM nephrotoxic serum which had been prepared as described previously (96); control animals received 0.1 ml of normal

rabbit serum. The kinetics of leukocyte infiltration and glomerular injury associated with injection of this antiserum have been reported previously (97). In the present study animals were sacrificed at 2 (n=4) and 7 days (n=4) following the administration of nephrotoxic serum. The latter group of animals received either 4.8 μg intravenous IL-4 (obtained from the conditioned media of a CHO-K1 cell line transfected with rat IL-4 cDNA and quantified by ELISA, Biosource, U.K.) (n=4) or media from untransfected parental CHO-K1 cells (n=4) twice daily intravenously from day 4 to day 7 post-induction of nephritis. For all animals glomeruli were isolated by standard sieving techniques (97). Samples of kidney were either placed in optimal cutting temperature (OCT) compound and snap frozen in liquid nitrogen cooled isopentane or used for the isolation of glomeruli by standard sieving techniques.

RT-PCR

Transformed RGEC, primary rat mesangial cell or glomerular total RNA was isolated using Trizol (Gibco) according to the manufacturers' instructions. Total RNA (2μg) was treated with DNase I and Oligo$(dt)_{12\text{-}18}$ was then used to generate first strand cDNA. Reverse transcription was carried out using Superscript II RNase H. Sequence-specific primer pairs were designed and obtained from Sigma-Genoys (Cambridge, UK) (Table 3.2) Amplification was as previously described, typically 94^0C for 3 minutes; 35-40 cycles of 94^0C for 30 seconds (denaturing), 55^0C for 1 minute (annealing) and 72^0C for 1 minute (extension); followed by a final extension step of 72^0C for 7 minutes. cDNA samples were subjected to parallel PCR reactions with primers for GAPDH/β-actin to control for equivalency of loading. The presence of genomic DNA was determined by control reactions in which amplification was

conducted in complete reaction mixture lacking template cDNA or with RNA samples from RT reactions carried out in the absence of Superscript II. PCR products were visualised by ethidium bromide staining following electrophoresis on 1.2% agarose gels. Subsequent quantification for each gene studied in comparison to the GAPDH loading control was conducted using the GelWorks 1D software package (UVP, Cambridge UK).

Rat Gene	Annealing Temp. °C	Product Length, bp	Sequence P1= Primer 1 P2= Primer 2
SOCS			
SOCS-1	55	273	**P1** 5'-TCC GCT CCC ACT CTG ATT AC-3' **P2** 5'-CGA AGA GGC AGT CGA AGG T-3'
SOCS-2	55	333	**P1** 5'-AAA TTA AAA GAG GCG CCA GA-3' **P2** 5'-AAT GCT GAG TCG GCA GAA GT-3'
SOCS-3	55	200	**P1** 5'-GCT ACC CTC CAG CAT CTT TG-3' **P2** 5'-GGC TGG ATT TTT GTG CTT GT-3'
CIS-1	55	201	**P1** 5'-TCT CCT ACC TCC GGG AAT CT-3' **P2** 5'-CCA GTC GGA AGC TAG AGT CG-3'

Table 3.2 Rat SOCS primers.

Immunohistochemistry

Paraffin sections were used for immunohistochemical staining for SOCS-3 and CIS-1. Goat polyclonal SOCS-3 and CIS-1 antibodies (Santa Cruz Biotechnology) were used at a concentration of 5μg/ml, overnight at 4°C. In both cases detection of primary antibody was via biotinylated horse anti-goat IgG (1:500, Vector Labs) and streptavidin-biotin-peroxidase conjugates (1:500, Boehringer Mannheim).

3.7.3. Results

As has previously been stated, cytokine-dependent upregulation of SOCS exhibits a marked cell-type and tissue-type specificity. For this reason, although a number of the cytokines used in the present study have been shown to upregulate various SOCS in haematopoietic tissue and cell lines, liver and pituitary it was necessary to determine the pattern of SOCS upregulation in response to these mediators in renal cells. The role of resident mesangial cells in glomerular inflammation is well established, as is the pivotal role of endothelial activation in many inflammatory conditions. These two cell types were therefore chosen as a cell culture model in which to begin the investigations. Initial experiments were carried out to determine which SOCS species were basally expressed in these cells. RT-PCR analysis using sequence specific primers for CIS-1, SOCS-1, -2 and –3 was carried out on total mRNA isolated from RGEC and rat mesangial cells. In both cell types SOCS-3 and CIS-1, but not SOCS-1 and SOCS-2, were found to be constitutive under basal conditions. Following cytokine stimulation SOCS 3 and CIS-1 expression in RGEC was upregulated, to varying degrees, in response to the cytokines IL-1β, IL-4, IL-6 and TNF-α and IFN-γ (Fig 3.12, 3.13). In contrast, no significant upregulation of SOCS-3 in response to these cytokines occurred in rat mesangial cells, while increases in CIS-1 mRNA were apparent only after stimulation with TNF-α. (Fig 3.14, 3.15). Expression of SOCS-1 and SOCS-2 in these two cell types was not induced in response to these cytokines.

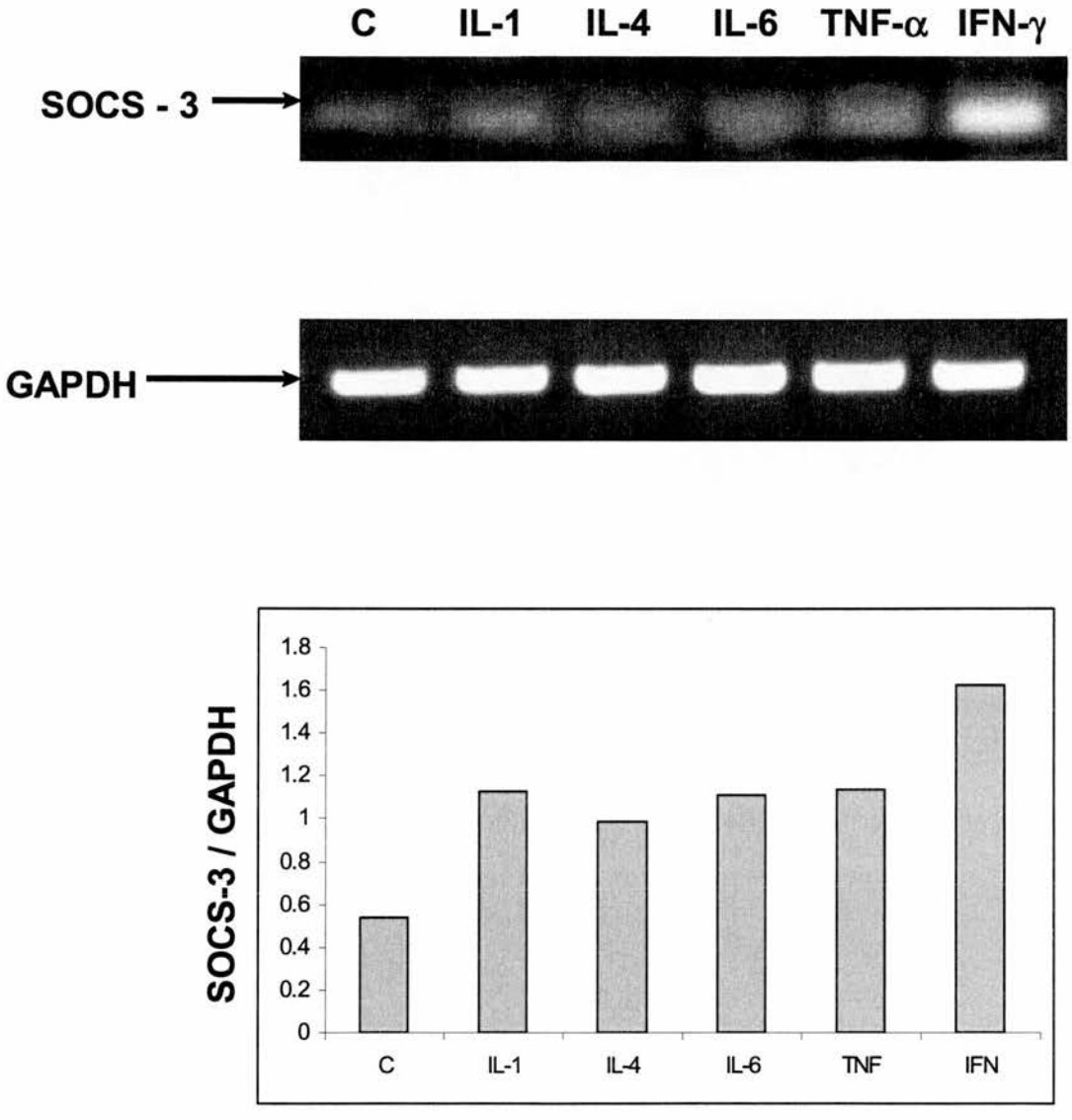


Figure 3.12. SOCS-3 expression in RGEC in response to cytokine stimulation.

SOCS-3 is expressed in RGEC under basal conditions (c=control). mRNA levels of SOCS-3 in RGEC are increased in response to the cytokines IL-1 (2 fold), IL-4 (1.8 fold), IL-6 (2.0 fold), TNF-α (2.1 fold) and IFN-γ (3 fold). Result shown is representative of three separate experiments (Appendix 1C).

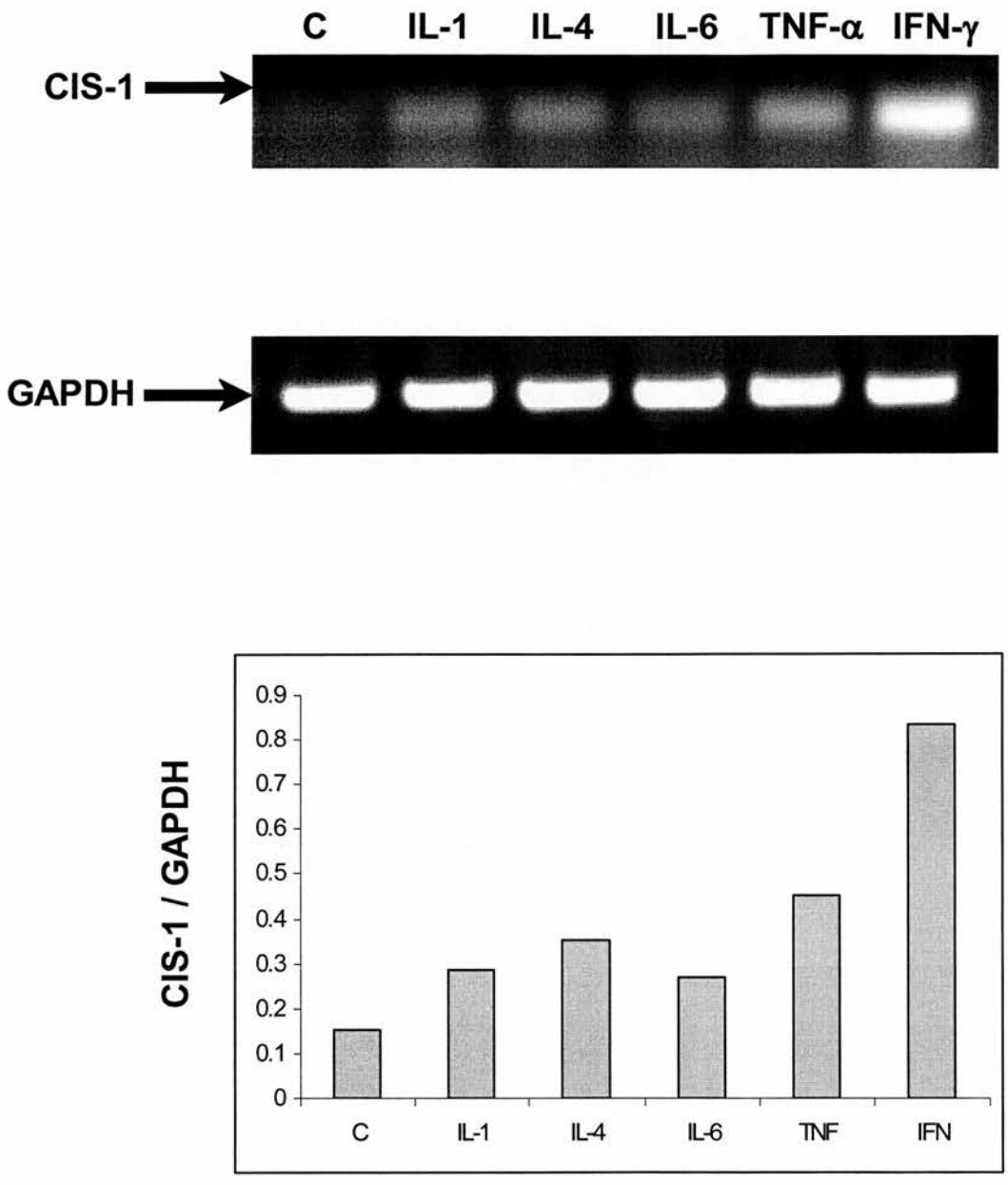


Figure 3.13. CIS-1 expression in RGEC in response to cytokine stimulation.

CIS-1 is expressed in RGEC under basal conditions (c=control). mRNA levels for CIS-1 in RGEC are increased in response to the cytokines IL-1 (1.9 fold), IL-4 (2.3 fold), IL-6 (1.8 fold), TNF-α (3 fold) and IFN-γ (5.5 fold). Result shown is representative of three separate experiments (Appendix 1C).

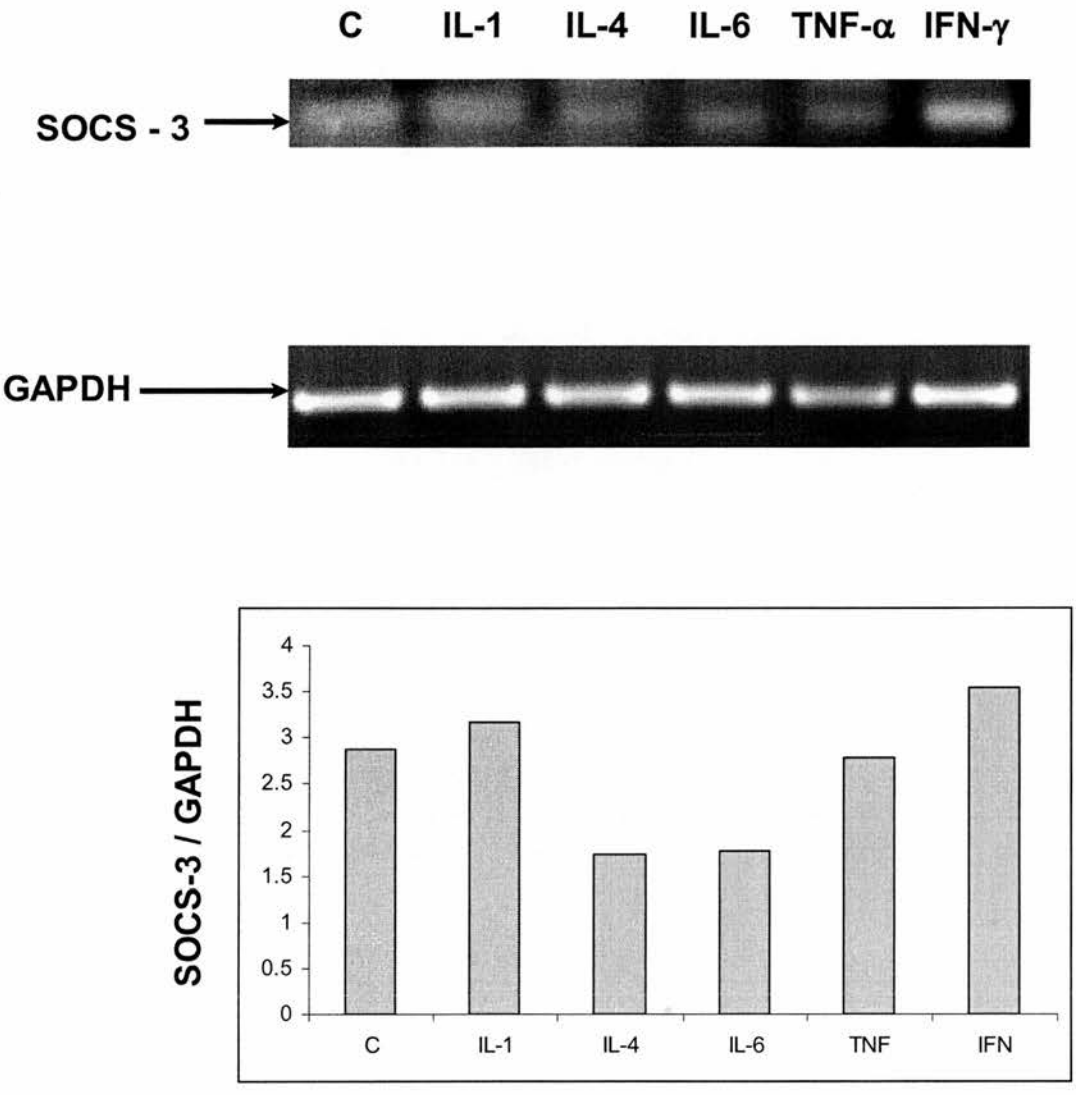


Figure 3.14 SOCS-3 expression in primary rat mesangial cells in response to cytokine stimulation.

SOCS-3 expression in primary rat mesangial cells occurs under basal conditions (c=control) and demonstrates no significant upregulation in response to the cytokines IL-1, IL-4, IL-6, TNF-α and IFN-γ. Result shown is representative of three separate experiments (Appendix 1C).

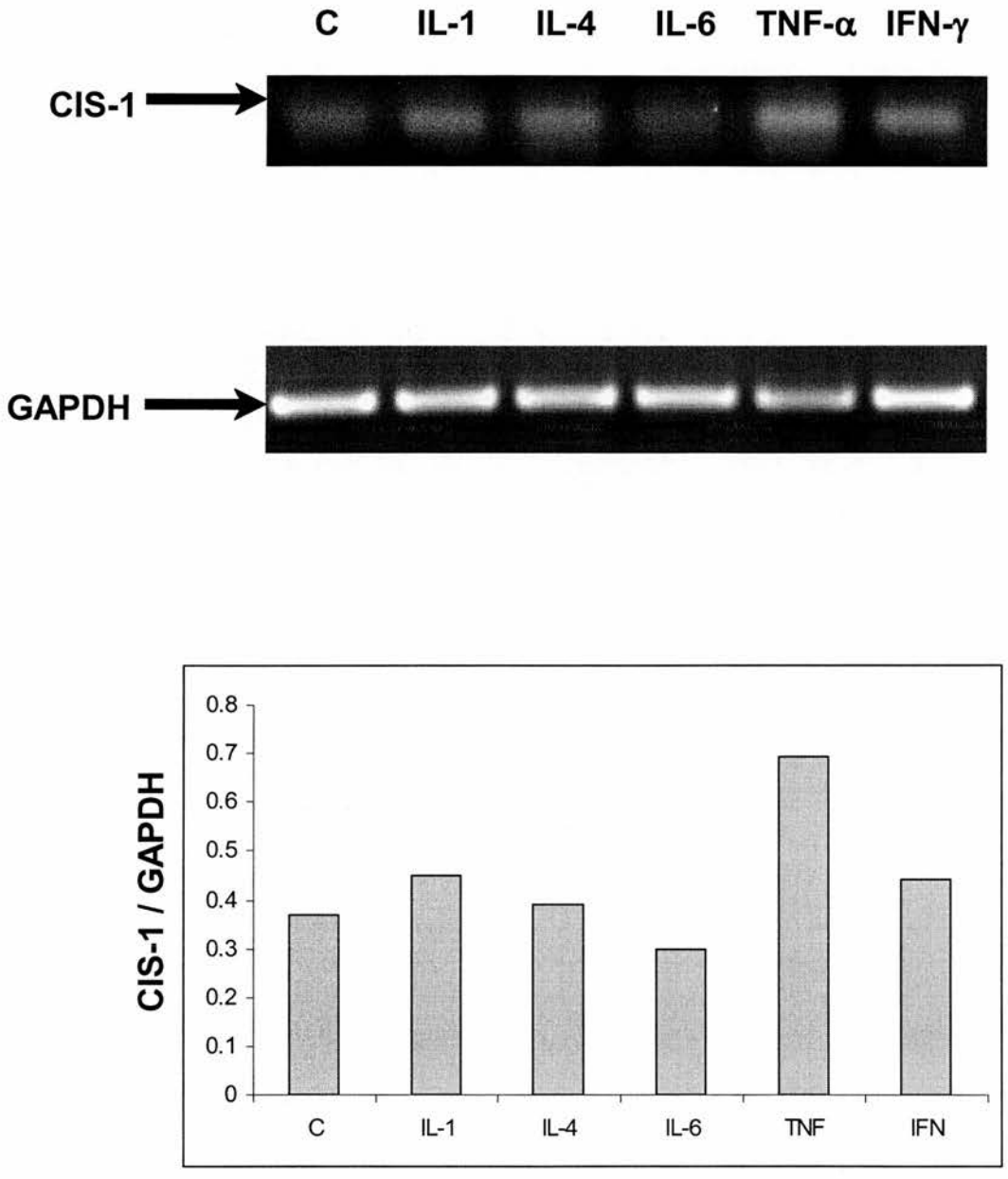


Figure 3.15 CIS-1 expression in primary rat mesangial cells in response to cytokine stimulation.

CIS-1 is expressed in primary rat mesangial cells under basal conditions (c=control). Levels of CIS-1 mRNA are increased in response to TNF-α (1.9 fold), but not in response to any other cytokine studied. Result shown is representative of three separate experiments (Appendix 1C).

Interferon-γ resulted in a marked upregulation of SOCS-3 expression (3 fold), while 3 and 5.5 fold increases in CIS-1 mRNA occurred in response to TNF-α and IFN-γ, respectively in these cells. Less dramatic increases in SOCS-3 and CIS-1 expression occurred in response to the other cytokines studied; SOCS-3 expression in response to IL-1β (2.0 fold), IL-4 (1.8 fold), IL-6 (2.0 fold) and TNF-α (2.1 fold): CIS-1 expression in response to IL-1 (1.9 fold), IL-4 (2.3 fold) and IL-6 (1.8 fold). In contrast, while CIS-1 mRNA in mesangial cells increased 1.9 fold following TNF-α treatment, no significant increases in either CIS-1 or SOCS-3 expression in these cells occurred in response to any other cytokine studied. Obviously in view of the limitations of semi-quantitative RT-PCR, these results will be confirmed using Northern analysis.

In summary, increases in the expression of SOCS-3 and CIS-1 in RGEC appeared to occur in response to both pro- (IL-1β, TNF-α and IFN-γ) and anti-inflammatory (IL-4 and IL-6) cytokines. Rat mesangial cells appeared less responsive to cytokine-induced SOCS-upregulation, with only TNF-α producing a modest increase in CIS-1 expression in these cells.

Other investigators in our group have examined the role of cytokines in the modulation of SOCS expression in another cell culture model – an SV-40 transformed murine mesangial cell line, MES 13 (ATCC, Maryland, USA) (98). MES 13 cells also express SOCS-3 and CIS-1 under control conditions while increases in SOCS-3 and CIS-1 mRNA occur in response to TNF-α and IL-1β stimulation of these cells. IFN-γ has no significant effects on cytokine-induced

SOCS-3 and CIS-1 upregulation in these cells. The anti-inflammatory cytokines IL-4 and IL-13 increased expression of CIS-1, but not SOCS-3. This study also examined whether IL-6 and its family members could modulate SOCS expression in MES 13 cells. In brief, significant upregulation of SOCS-3 and CIS-1 occur in response to IL-6 and the related cytokines, LIF and IL-11.

Since studies in RGEC (and previously in MES 13) cells indicated that SOCS were upregulated *in vitro* in response to both pro- and anti-inflammatory cytokine stimulation, it was unclear whether SOCS expression levels *in vivo* would correlate with the extent or the resolution of inflammation. In order to assess this, the glomerular expression of SOCS-2, -3 and CIS-1 in a rat model of crescentic nephritis was determined.

Total glomerular RNA was isolated from control animals and from those animals to whom nephrotoxic serum had been administered 2 and 7 days previously. Analysis of SOCS expression was by means of RT-PCR using sequence specific primers (Table 3.2). As can be seen, SOCS-3 and to a variable extent CIS-1 were basally expressed in normal rat glomeruli. By day 7 post-induction of GN, however, CIS-1 and SOCS-3 expression were markedly increased over control levels in these animals (Fig. 3.16, 3.17).

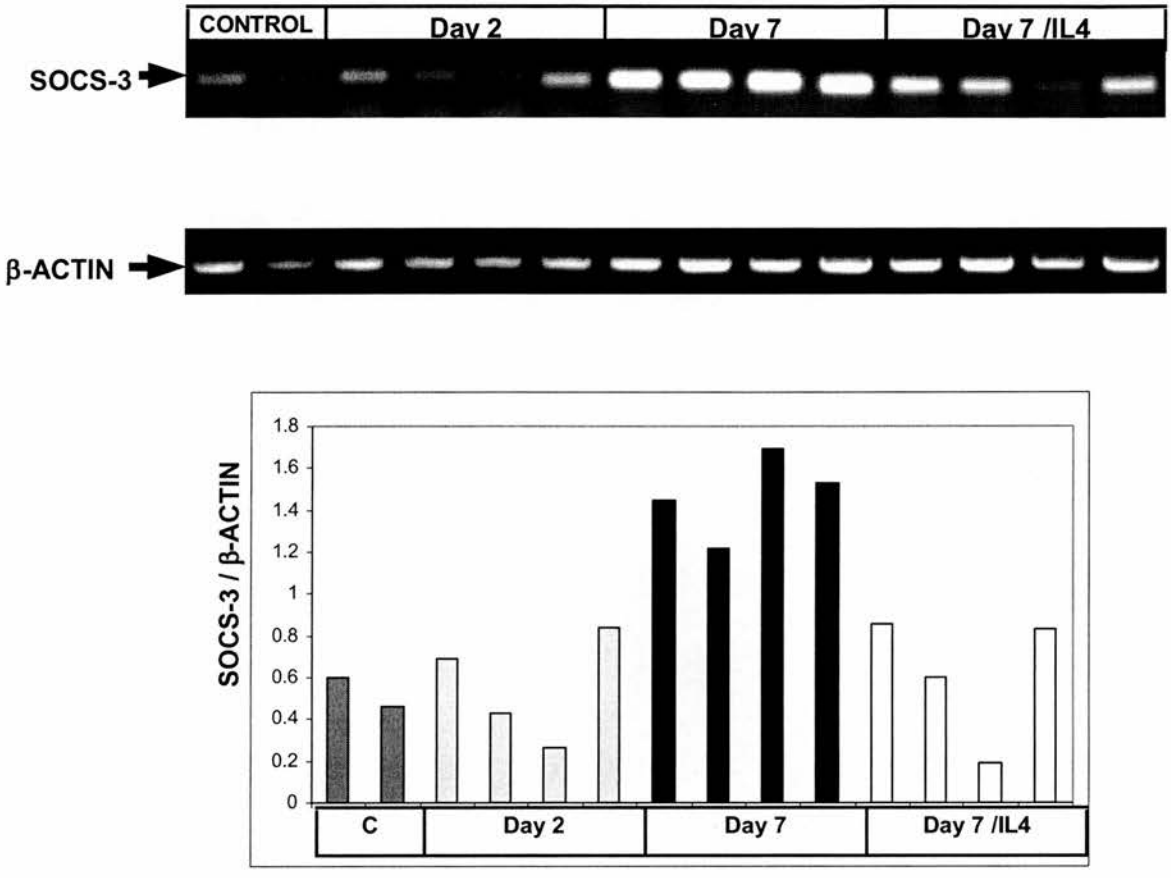


Figure 3.16. SOCS-3 expression in a rat model of crescentic nephritis.

SOCS-3 was basally expressed in normal rat glomeruli. By day 7 post-induction of GN, however, SOCS-3 expression was markedly increased over control levels. IL-4 administration led to an attenuation in disease activity and glomerular expression of SOCS-3 was also reduced in animals receiving IL-4

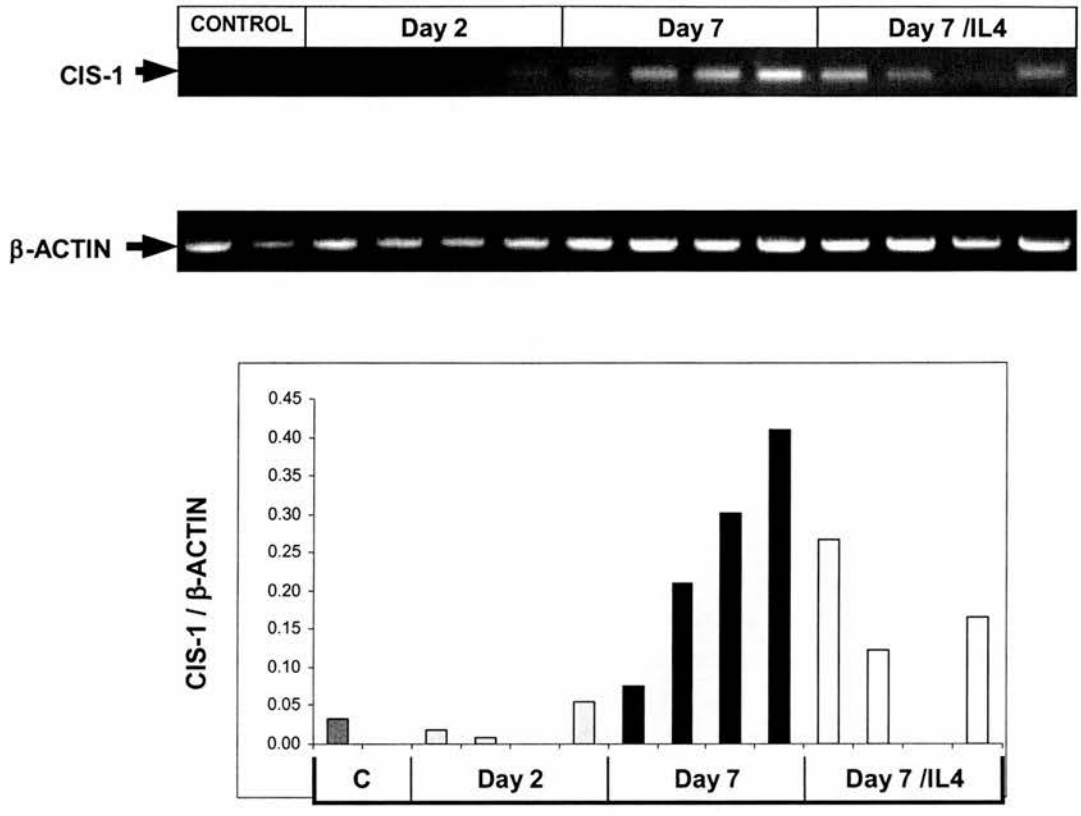


Figure 3.17. CIS-1 expression in a rat model of crescentic nephritis.

Basal expression of CIS-1 in rat glomeruli varied between barely apparent to completely absent from animal to animal. By day 7 post-induction of GN, however, CIS-1 expression was markedly increased over control levels. IL-4 administration led to an attenuation in disease activity and glomerular expression of CIS-1 was also reduced in animals receiving IL-4.

In order to assess whether treatment of GN with IL-4, having delayed the administration of this cytokine until inflammation was established, would have any effect on glomerular expression of CIS and SOCS-3, a group of rats were given i.v. IL-4 twice daily from day 4 - day 7. IL-4 administration led to an attenuation in disease activity (as indicated by significantly reduced proteinuria and fibroid necrosis when compared to day 7 nephritic animals receiving no cytokine therapy (99). Glomerular expression of CIS and SOCS-3 was also reduced in animals receiving IL-4 (Fig. 3.16, 3.17).

On the basis of these results, immunohistochemical staining for SOCS-3 and CIS-1 was carried out on paraffin sections of the kidneys from these animals. To date staining for CIS-1 has not been successful and work is ongoing overcome these technical difficulties. In the normal kidney, SOCS-3 is present in distal tubules, but undetectible in glomeruli (Fig. 3.18a and b). By day 2 faint glomerular SOCS-3 staining is apparent with definite glomerular staining occurring at day 7 (Fig 3.18c and d). Staining appeared to occur in cells with the morphology of macrophages, however, no double-staining techniques were used in this study, so the possibility that some SOCS-3 staining is occuring in intrinsic glomerular cells cannot be excluded. Immunohistochemistry revealed no clear differences between SOCS expression at day 7 in the IL-4 treated rats and in those animals receiving no cytokine therapy (Fig. 3.18e and f).

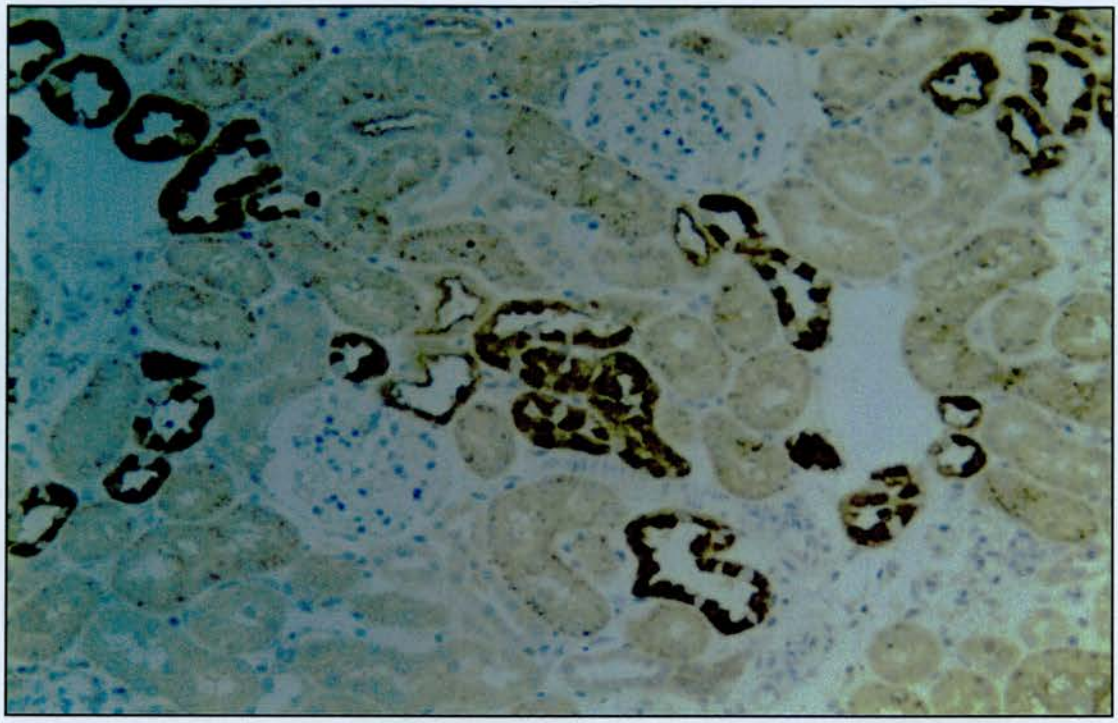


Figure 3.18a SOCS-3 staining in normal kidney

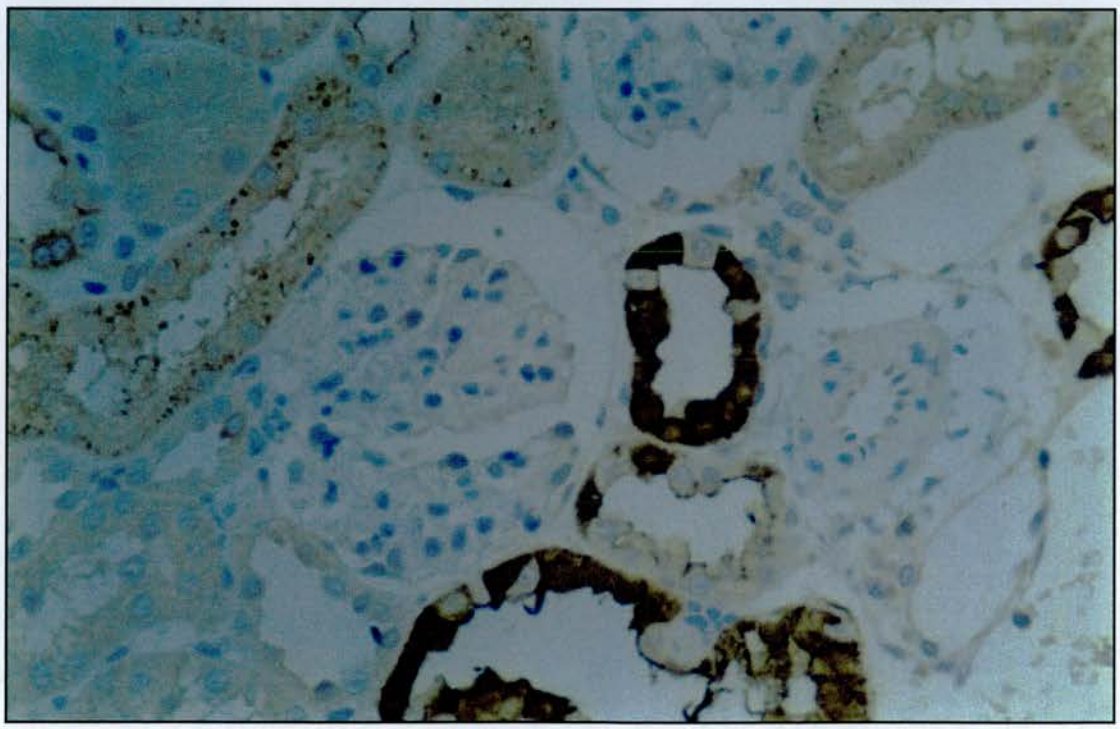


Figure 3.18b SOCS-3 staining in normal kidney

In the normal kidney SOCS-3 is present in distal tubules but undetectible in glomeruli.

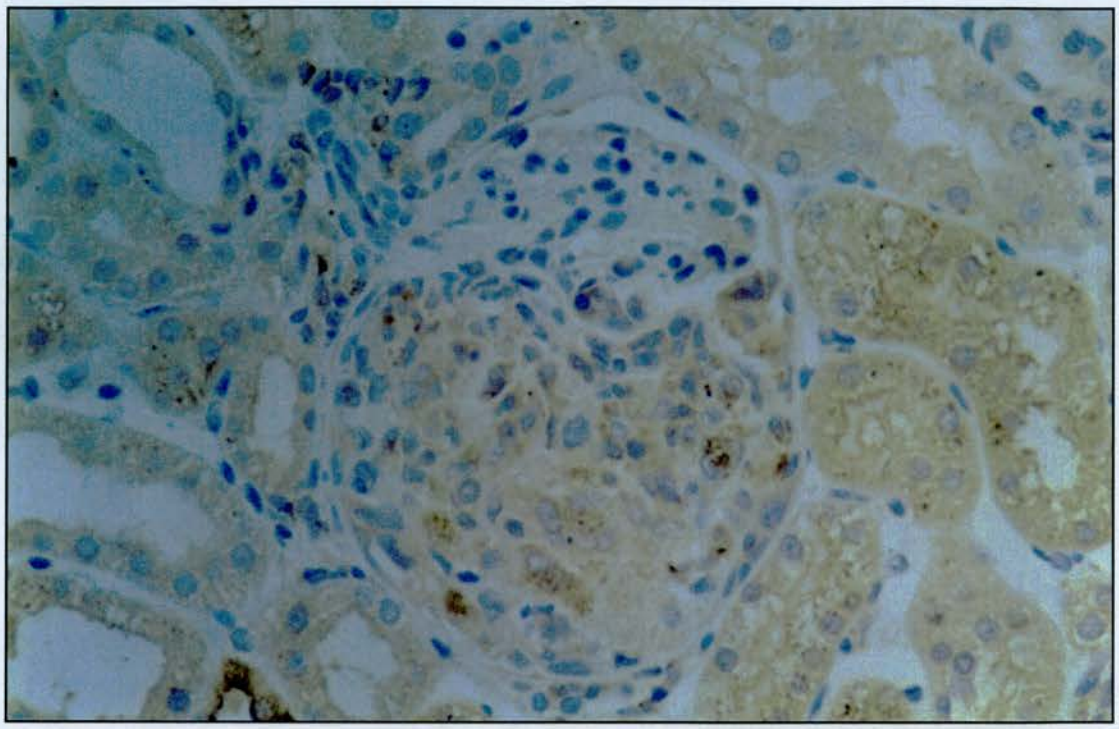


Figure 3.18c SOCS-3 staining at day 7 in crescentic nephritis.

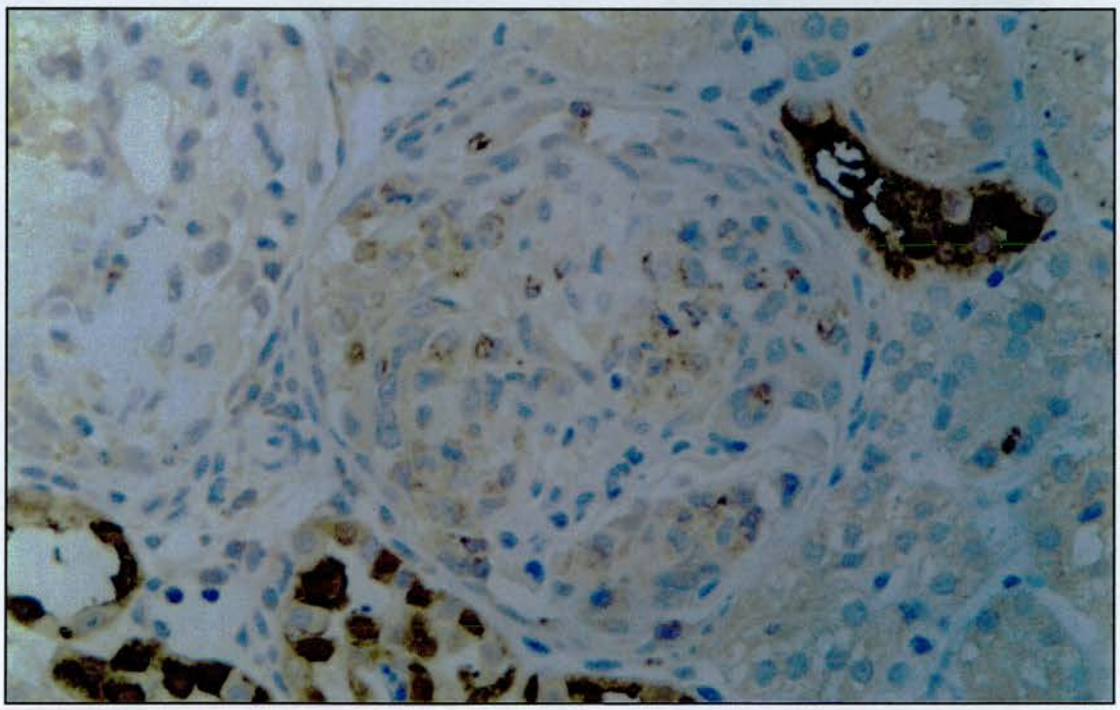


Figure 3.18d SOCS-3 staining at day 7 in crescentic nephritis

In addition to SOCS-3 staining in distal tubules, definite staining occurs in glomeruli at day 7 in crescentic nephritis.

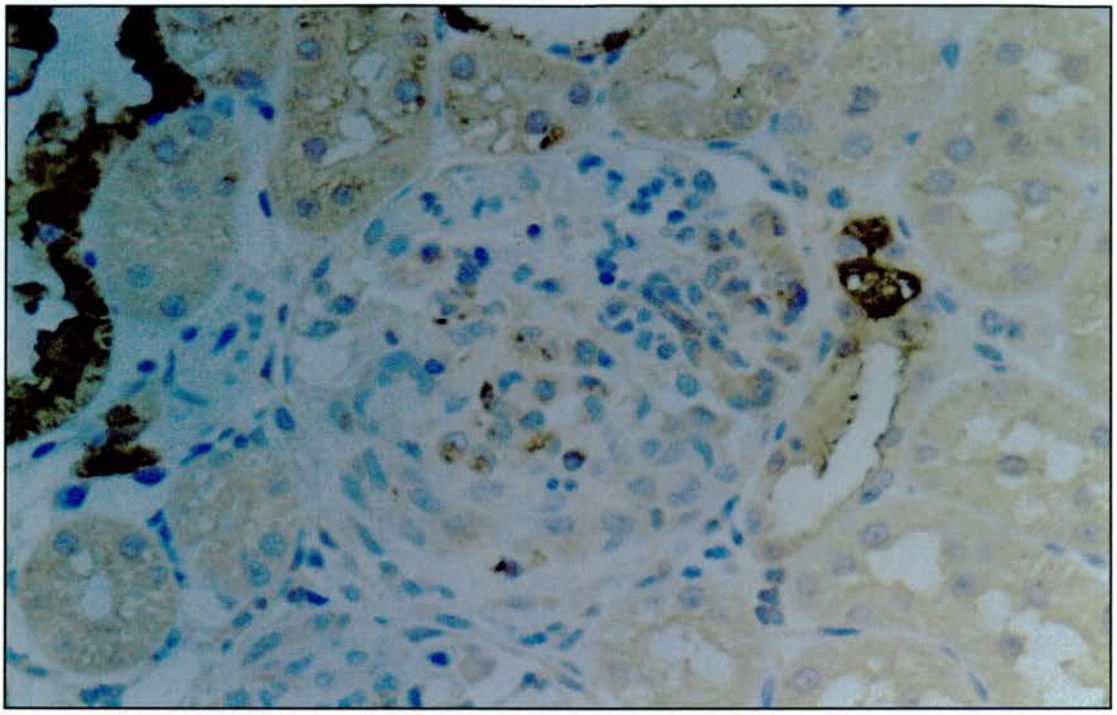


Figure 3.18e. SOCS-3 staining at day 7 in crescentic nephritis following IL-4

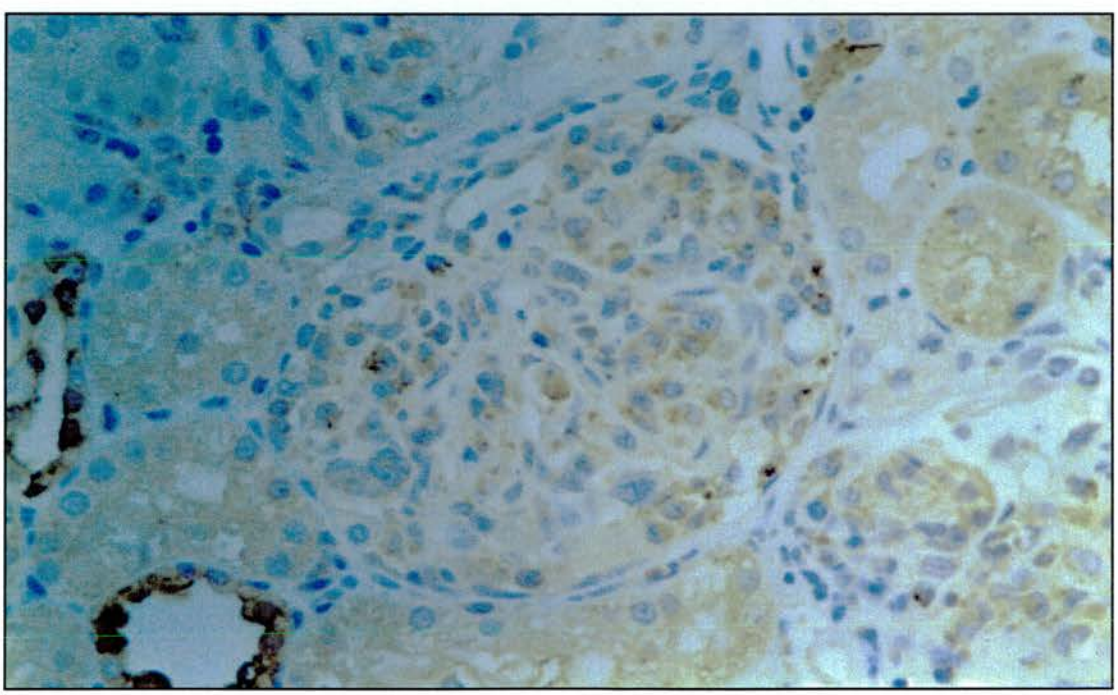


Figure 3.18f. SOCS-3 staining at day 7 in crescentic nephritis following IL-4

Immunohistochemistry revealed no apparent differences between SOCS-3 expression at day 7 in the IL-4 treated rats and in those animals receiving no cytokine therapy.

3.7.4. Discussion

The current study attempted to define the role of SOCS in renal inflammation. *In vitro* studies employing various renal cells (RGEC, MES 13) determined that upregulation of these species was a consequence of both pro-inflammatory (TNF-α, INF-γ and IL-1β) and anti-inflammatory (IL-4 and IL-6) cytokine stimulation, suggesting that CIS-1 and SOCS-3 upregulation was a pleiotropic negative regulatory mechanism whereby cytokine-dependent Jak/STAT signaling was blunted. In agreement with these findings, *in vivo* investigations employing a rat model of nephrotoxic nephritis found that SOCS-3 and CIS-1 upregulation correlated with disease activity. Taken together these *in vitro* and *in vivo* findings imply that, in the scenario of renal inflammation, the expression level of a given SOCS species is likely to reflect the total load of cytokines (both pro- and anti-inflammatory) within the site of inflammation capable of upregulating its expression.

As has been previously stated, both the specificity of SOCS expression and the kinetics which this expression follows appear to be regulated at the transcriptional level in a cell and tissue-dependent fashion. Cytokine-receptor interaction, the particular JAKS which this activates, or indeed the STAT dimers which are consequently utilised to initiate transcription, do not appear to define SOCS expression with the same certainty as has been evidenced for other cytokine-inducible genes. For this reason it was necessary to define the pattern of basal and induced SOCS expression in the *in vitro* and *in vivo* models used in the current study. Interestingly, the basal expression of SOCS-3 characteristic of all the cell types studied here was also found in glomerular RNA samples. However, while CIS-1 expression was constitutive in these cell types, normal rat glomeruli did not

consistently express this transcript. In our hands basal expression of CIS-1 in rat kidney - both cortex and purified glomeruli - varied (between barely apparent to completely absent) from animal to animal. While it has been reported that murine kidney exhibits pronounced basal CIS-1 expression (61), it also appears that unstimulated CIS-1 expression in murine tissues (liver, thymus and, to a lesser extent, kidney) is gender-dependent, with female animals exhibiting significantly enhanced levels (60). Since male rats were used in the present study, this remains as a possible explanation for the observed variability.

While only significant TNF-α induced upregulation of CIS-1 could be demonstrated in rat mesangial cells, both CIS-1 and SOCS-3 were determined to be the relevant agonist-inducible SOCS in RGEC and MES 13. Treatment of these cells with various pro- and anti-inflammatory cytokines resulted in the upregulation of one, other or both of these transcripts. Similarly, CIS-1 and SOCS-3 were amplified from nephritic glomeruli cDNA. It must be pointed out, however, that while mesangial cells in particular may be taken as an indicator of the responses of the predominant resident cell in the glomerulus, in the scenario of renal inflammation, the situation becomes much more complex due to the accumulation of mononuclear cells. Regardless, the data demonstrate a broad correlation between the pattern of basal and inducible SOCS expression in an *in vitro* and *in vivo* model of renal inflammation. This parallels other studies in which the *in vitro* expression of a SOCS species in tissue-derived cells corresponded to its *in vivo* expression in the originator organ; SOCS-3, for example, was found to be the predominant basally-expressed and LIF-induced species in both a pituitary tumor-derived corticotroph cell line and in murine pituitary *in vivo* (87).

WKY rats have been shown to be reliably susceptible to the induction of severe crescentic GN in response to a single i.v. dose of anti-GBM containing serum (97, 100). This model is, therefore, consistent and reproducible since the requirement for pre-immunisation associated with most animal models of accelerated GN is removed and potential variations in disease progression arising from differences in individual immune responses are no longer an issue. SOCS expression was investigated over seven days following the administration of nephrotoxic serum, since it was reasoned that this time course incorporated the initial renal assault, the primary immunologic response and the peaks of both the heterologous and autologous phases of the disease.

It has been shown that a number of leukocyte adhesion molecules, pro-inflammatory cytokines and chemokines facilitate the infiltration of glomeruli (and/or tubulo-interstitum) by mononuclear cells in experimental nephrotoxic nephritis (100-104). Nephrotoxic GN in WKY rats is associated with infiltration of leukocytes (predominantly monocytes/macrophages and CD8+ lymphocytes) within 2.5 hr p. i., reaching a peak between day 4 and day 8. CD8+ cells (which at their day 4 maximum represent 20-25% of the total infiltrate) appear to play a pivotal role in the pathogenesis of this model. Depletion of CD8+ cells has been shown to significantly reduce glomerular monocyte/macrophage accumulation (100, 105) and to completely prevent proteinuria and crescent formation (105). It has been speculated that CD8+ cells act to promote monocyte/macrophage accumulation and, hence, the pathogenesis of GN via their production of pro-inflammatory and chemotactic cytokines. CD8+ cell depletion has been shown to reduce the early glomerular expression of a number of cytokines and it has been proposed that CD8+-derived

mediators facilitate monocyte/macrophage infiltration in part through their induction of glomerular cytokine/chemokine and intercellular adhesion molecule-1 (ICAM-1) expression. Indeed, glomerular ICAM-1 levels are markedly reduced in CD8+depleted nephritic WKY rats, in tandem with a reduced monocyte/macrophage infiltrate (100). The temporal profile of glomerular pro-inflammatory cytokine (namely TNF-α, IL1-β, INF-γ) and chemokine induction in the WKY model parallels that of ICAM-1; expression is apparent within 1 hr and significantly elevated at day 3. It is not surprising therefore, that the current study found CIS-1 and SOCS-3 levels to be maximal at day 7, since logic dictates that stimulated levels of these species should kinetically trail the peak of cytokine expression.

Glomerular expression of SOCS-3 and CIS-1, as detected by RT-PCR, was reduced in animals receiving IL-4. In contrast, no definite difference in the glomerular expression of SOCS-3 could be detected using immunohistochemistry. Immunoperoxidase staining is, however, a relatively insensitive technique for the assessment of moderate expression differences and therefore, small changes between the treated and untreated animals might not be readily apparent. The ability to detect basal glomerular SOCS-3 expression in normal glomeruli by semi-quantitative RT-PCR but not immunohistochemistry supports this contention.

A detailed immunohistological analysis of renal sections in this model has been previously performed by other investigators. These studies indicated that in animals receiving IL-4 the number of infiltrating macrophages were not reduced over control. There was, however, significantly fewer iNOS positive cells and markedly reduced staining for sialoadhesin, markers of macrophage activation (99). These results

suggest that the upregulation of SOCS-3 and CIS-1 at day 7 arises as a consequence of increased production and activity of cytokines within the inflammatory site. Conversely, the blunting of macrophage activity following IL-4 administration is likely to result in reduced ambient cytokine levels and hence reduced SOCS-3 and CIS-1 expression.

In summary the up-regulation of SOCS, namely SOCS-3 and CIS-1, in renal cells *in vitro* has been demonstrated in response to both pro- and anti-inflammatory cytokines. In keeping with these observations, increases in SOCS-3 and CIS-1 have been demonstrated in rat nephrotoxic nephritis. In this model it appears that SOCS expression correlates with disease activity, providing evidence that these proteins may represent a pleiotropic negative feedback mechanism by which the JAK/STAT-dependent actions of cytokines are blunted. To-date, SOCS expression has not been reported in other inflammatory renal diseases. However, it will be intriguing to determine their pathophysiological role and potential therapeutic targets in cytokine-driven renal disease.

3.8. SOCS expression in cardiac transplant rejection and correlation with cytokine levels and immune cell infiltration

3.8.1. Introduction

Having studied SOCS in autoimmune inflammation, the profile of SOCS expression in an animal model of alloantigen-driven inflammation was next examined. Since I am a cardiologist an animal model of experimental acute cardiac transplant rejection was chosen.

The development of microsurgical techniques has provided a potent tool in the study of whole organ allograft immunobiology in rodents. Studies in such models have greatly expanded the knowledge of transplant immunology and have been a driving force in the development of the now commonly used various immunosuppressive regimes (106). One of the most technically feasible models that facilitates studies into cardiac rejection is the heterotopic cardiac allograft (107). Here the donor graft is transplanted infrarenally into the pelvis of the recipient by anastomosis to aorta and inferior vena cava and the native heart is left in situ. A cervical accessory model with anastomosis to recipient carotid artery and jugular vein also exists but this is used less frequently. In such models the transplanted heart can be easily palpated, with rejection defined as complete cessation of palpable heart beat (and confirmed at laporatomy). Loss of pulsation in the first 48 hours however is regarded as an indicator of operative failure and can be confirmed by the presence of thrombus at anastomotic sites. Once the animal is sacrificed both native and donor heart can be retrieved ensuring the easy availability of suitable controls in these studies. The degree of MHC mismatch between donor and recipient, in addition to the

administration (or not) of immunosuppressive therapy, determines whether the model is one of acute or chronic rejection (108).

Heterotopic cardiac allografts exchanged across the MHC in terms of complete Class I and Class II mismatch provide a highly reproducible and rigidly controlled model for studies of acute rejection. Immunohistochemistry in such models has confirmed progressive mononuclear cell (CD4+, CD8+ and macrophages) infiltration into allograft tissue (109, 110). In addition there is severe diffuse loss of integrity of the microvascular endothelium providing strong evidence that the allograft microcirculation is a central target of graft destruction. The central role of T cells in acute graft rejection has been shown conclusively in athymic mice, which fail to produce mature T cells (111). These mice accept grafts from syngeneic, allogeneic or even xenogeneic donors, without evidence of rejection. The CD4+ and CD8+ T cell subset are independently capable of mediating graft rejection. Studies have shown that administration of monoclonal antibody to either CD4+ or CD8+ cells is ineffective in preventing graft rejection confirming the functional overlap of these lymphocyte subsets (112). Similarly, the experimental elimination of either MHC Class I or Class II molecules by gene inactivation using homologous recombination ("knock-out") technology in mice has shown that grafts expressing only Class I (predominantly recognised by CD8+ T cells) or Class II (predominantly recognised by CD4+ T cells) are both readily rejected (113).

The first experimental studies which analysed the molecular pathways involved in acute rejection and graft acceptance provided evidence that these mechanisms were dominated by intragraft production of either Th1 or Th2 cytokines, respectively.

Studies to date in these models have demonstrated the consistent upregulation of various cytokines and chemokines. The temporal nature of these responses varies slightly, depending on the animal used and MHC mismatch.

It has become evident using genetically manipulated animals, however, that rejection and acceptance are not exclusively restricted to the type1/type 2 dichotomy. IL-2 and IFN-γ "knockout" mice reject their transplants in the presence of Th2 cytokines and IL-4 knockout mice accept their transplants in the presence of Th1 cytokines (114). In addition to the upregulation of cytokines and chemokines, the upregulation of adhesion molecules, MHC molecules, complement and the molecules implicated in cell-mediated cytotoxicity (perforin, granzyme) have been demonstrated in these models and evidence provided for their functional roles in the allogeneic immune response (115-119).

Mechanisms of immunosuppression

In the last decade, aided by such work in experimental transplant settings, there has been remarkable developments in the field of immunosuppression with the result that the transplant physician now has an expanding portfolio of immunosuppressive agents for the prevention and treatment of rejection (120). Most regimens employ a corticosteroid which blocks T cell activation and proliferation. Azathioprine also blocks lymphocyte proliferation following antigenic stimulation, possibly by blocking DNA replication. Mycophenolate mofetil is a novel immunosuppressive drug which also has a potent cytostatic effect (predominantly on lymphocytes), again by interfering with DNA synthesis (121). The demonstration in some experimental models of its ability to inhibit vascular smooth muscle proliferation may offer

potential in the treatment of chronic rejection. Cyclosporin, a small cyclic peptide of fungal origin, remains the basis for all current immunosuppressive protocols. By blocking the Ca^{2+} dependent component of the T cell receptor signal transduction pathway, it blocks the expression of cytokines produced by T cells including IL-2, IL-3, IL-4, IFN-γ and TNF-α (122). It does not, however, interfere with IL-1 or TNF production by APC or macrophages. FK 506, although differing in structure to cyclosporin, acts through the same final common pathway to block the induction of cytokine RNA and indirectly inhibit T cell proliferation (123). Rapamycin is a potent immunosuppressive agent which is currently undergoing clinical trials. In contrast to cyclosporin and FK 506 which inhibit an early signal in T cell activation, rapamycin inhibits late signals in T cell activation by preventing phosphorylation of p70 kinase in the CD28 co-stimulatory and IL-2R signal transduction pathways (124). Combination therapy with cyclosporin and rapamycin has been shown to synergistically increase immunosuppression in experimental models.

Administration of polyclonal anti-lymphocyte globulin results in the elimination of circulating T cells and resolution of acute graft rejection episodes; however, in addition to profound immunosuppression, the heterogeneity of the antibody mixture can lead to variable efficacy as well as adverse reactions. The development of monoclonal antibodies to T cell surface molecules offers the advantage of more predictable therapeutic agents (120). The monoclonal antibody OKT3 which is routinely used in clinical practice binds the T cell receptor and blocks both CD4+ and CD8+ T cell function. A number of other antibodies such as anti-leukocyte function-associated antigen-1 (LFA-1), anti-ICAM-1, anti-CD4 and anti-CD25 have

been shown to reduce rejection in animal models and are currently being tested in clinical trials (125).

Tolerance induction

The incidence of organ rejection, and of death from infection, have decreased over time, primarily as a result of improvements in immunosuppression and in the prevention and treatment of infection. These advances not with-standing, immunological rejection and its sequlae – toxicity from immunosuppressive regimes and graft dysfunction – remain the major cause of morbidity and mortality in transplant patients. The increased incidence of cancers such as lymphoma secondary to prolonged immunosuppression is also a concern. The ultimate goal of clinical transplantation is to induce donor-specific unresponsiveness in the recipient while maintaining responsiveness to donor antigens. Most approaches to tolerance induction to an allograft in the experimental model involve pretreatment or treatment with antigen in one form or another, usually in association with immunosuppressive therapies. Intrathymic immune modulation with donor antigens in the form of donor cells, purified MHC antigens, synthetic allopeptides, in addition to donor specific blood transfusion, have been demonstrated to be effective means of inducing long term allograft survival in various experimental systems (126-130). The exact mechanisms induced using this approach are not yet well defined. An alternative strategy has been to inhibit the costimulatory signal during T cell encounter with donor antigen and induce non- specific tolerance. Blockade of CD28-B7 binding by CTLA4Ig in combination with donor specific lymphocytes, indefinitely prolonged graft survival in a heterotopic transplant model (131). Recently in human bone marrow transplantation, donor bone marrow was co-cultured with irradiated cells

from the recipient in the presence of CTLA4-Ig and then transfused into the recipient following conventional myeloablation (132). This resulted in anergy to alloantigens with a low risk of graft versus host disease, while responsiveness to other unrelated alloantigens was unaffected. The widespread applicability of this procedure to bone marrow transplantation, and indeed other organ transplantation, remains to be seen.

In summary then, allograft rejection, both acute and chronic, represents the end result of a complex set of immunological processes arising from the recognition of non-self antigens on transplanted tissues. The alloreactive phenotype is discussed in detail in section 1.3.5. Cytokines sustain and amplify the allograft-directed immune response by promoting lymphocyte activation, increasing adhesion molecule expression, and regulating MHC expression. Cytokine elaboration, and consequently cytokine-activated JAK/STAT mobilisation, is therefore a critical determinant of graft rejection. The *s*uppressors *o*f *c*ytokine *s*ignalling (SOCS) proteins function as negative regulators of cytokine-activated JAK/STAT signal transduction. Animal heterotopic cardiac allografts exchanged across the MHC in terms of complete Class I and Class II mismatch provide a highly reproducible and rigidly controlled model for studies of acute rejection. The aim of this study therefore was to examine the expression of SOCS in this experimental setting. In addition, the temporal expression of cytokines and leukocyte trafficking determinants were also investigated in this model.

3.8.2. Materials and Methods

Animals and grafting techniques

Inbred Lewis (LEW $RT1^L$) and Wistar-Furth (WF $RT1^U$) rats weighing 200-250g were purchased from Harlan Sprague-Dawley (Indianapolis, IN). LEW to WF heterotopic cardiac allografting was performed as previously described (107). Donor hearts were transplanted to the recipients abdomen by anastomising donor and recipient aorta, and donor pumonary artery to inferior vena cava. These cardiac allografts show an irreversible rejection 7-10 days post-transplantation. LEW to LEW transplants and native hearts served as isograft/ischaemia-reperfusion controls. Rejection was defined as complete cessation of palpable heart beat. Animals were sacrificed at days 1, 3, 5 and 7 post transplantation and both native (N) and donor heart (T) retrieved. A portion of each tissue was frozen in liquid nitrogen and stored at -80^0C for subsequent RNA extraction. The remaining portion was embedded in Cryo-gel (Instrumedics Inc., Hakensack, NJ) and immediately snap frozen in liquid nitrogen in preparation for immunohistologic studies.

Immunohistology

Cryostat sections were blocked with normal horse serum. Sections were also blocked for endogenous peroxidase using 0.3% H_2O_2 in methanol for 20 minutes. Sections were then stained with the following primary antibodies: CD 45 (mouse anti-rat CD 45, Serotec UK) 10-20 μg/ml for 1 hour at room temperature and ED1 (mouse anti-rat ED 1, Serotec UK) 10-20 μg/ml for 1 hour at room temperature. CD 45 (often referred to as common leukocyte antigen (CLA)) is a universal antigen present on all leukocytes, while ED-1 is a cytoplasmic antigen present in monocytes, macrophages

and dendritic cells. The secondary antibody in all cases was biotinylated anti-mouse IgG (affinity purified and not absorbed) 10 μg/ml for 30 minutes (Vector Labs, CA 94010). Detection system for all antibodies was Vectastain ABC System (Vector Labs, CA). Expression of both antigens on cardiac sections was graded semiquantitatively and reflected changes in the intensity of staining.

RT-PCR

Total RNA from cardiac tissue was isolated using Trizol reagent (Gibco Life Technologies, Paisley, Scotland) according to the manufacturers' instructions. Total RNA (2μg) was treated with DNase I and Oligo(dt)$_{12\text{-}18}$ was then used to generate first strand cDNA. Reverse transcription was carried out using Superscript II RNase H (all from Gibco). Sequence specific primer pairs were designed for each gene studied and obtained from Sigma-Genoys (Cambridge, UK). (Table 3.3). Amplification was as previously described, typically 94^{0}C for 3 minutes; 35-40 cycles of 94^{0}C for 30 seconds (denaturing), 55^{0}C-60^{0}C for 1 minute (annealing) and 72^{0}C for 1 minute (extension); followed by a final extension step of 72^{0}C for 7 minutes. cDNA samples were subjected to parallel PCR reactions with primers for GAPDH to control for equivalency of loading. The presence of genomic DNA was determined by control reactions in which amplification was conducted in complete reaction mixture lacking template cDNA or with RNA samples from RT reactions carried out in the absence of Superscript II. PCR products were visualised by ethidium bromide staining following electrophoresis on 1.2% agarose gels. Subsequent quantification for each gene studied in comparison to the GAPDH loading control was conducted using the GelWorks 1D software package (UVP, Cambridge UK).

Rat Gene	Annealing Temp. °C	Product Length, bp	Sequence P1= Primer 1 P2= Primer 2
SOCS			
SOCS-1	55	273	**P1** 5'-TCC GCT CCC ACT CTG ATT AC-3' **P2** 5'-CGA AGA GGC AGT CGA AGG T-3'
SOCS-2	55	333	**P1** 5'-AAA TTA AAA GAG GCG CCA GA-3' **P2** 5'-AAT GCT GAG TCG GCA GAA GT-3'
SOCS-3	55	200	**P1** 5'-GCT ACC CTC CAG CAT CTT TG-3' **P2** 5'-GGC TGG ATT TTT GTG CTT GT-3'
CIS-1	55	201	**P1** 5'-TCT CCT ACC TCC GGG AAT CT-3' **P2** 5'-CCA GTC GGA AGC TAG AGT CG-3'
GAPDH	6O	495	**P1** 5'-ACC ACA GTC CAT GCC ATC AC-3' **P2** 5'-TCC ACC ACC CTG TTG CTG TA-3'
CYTOKINES			
IL-1β	60	510	**P1** 5'-TCA TTG TGG CTG TGG AGA AG- 3' **P2** 5'-AGT TGG GGA ACT GTG CAG AC-3'
TNF-α	60	489	**P1** 5'-GCT CCC TCT CAT CAG TTC CA-3' **P2** 5'-AAG TAG ACC TGC CCG GAC TC-3'
IL-2	60	388	**P1** 5'-CAG CTC GCA TCC TGT GTT GCA C-3' **P2** 5-'CAC AGT TGC TGG CTC ATC ATC G-3'
IFN-γ	60	299	**P1** 5'-TTA CTG CCA AGG CAC ACT CA-3' **P2** 5'-ACT TGG CGA TGC TCA TGA AT-3'
IL-4	60	204	**P1** 5'-TCC TTA CGG CAA CAA GGA AC-3' **P2** 5'-TTG TGA GCG TGG ACT CAT TC-3'
IL-10	60	450	**P1** 5'-CCT GCT CTT ACT GGC TGG AG-3' **P2** 5'-TTC ATG GCC TTG TAG ACA CCT-3'
TGFβ	60	552	**P1** 5'- GTC AAC TGT GGA GCA ACA CG-3' **P2** 5'-TGG TTG TAG AGG GCA AGG AC -3'
CTGF	60	400	**P1** 5'-CTA AGA CCT GTG GAA TGG GC-3' **P2** 5'-CTC AAG ATG TCA TTG TCC CC-3'
CHEMOKINES			
GRO-α	60	180	**P1** 5'-AGA CAG TGG CAG GGA TTC AC-3' **P2** 5'-ACT TGG GGA CAC CCT TTA GC-3'
MCP-1	60	266	**Obtained from BioSource Int. CA.**
RANTES	60	176	
MIP-1α	60	202	
MIP-2	60	219	
CHEMOKINE RECEPTORS			
CCR1	60	503	**P1** 5'- AAA CCT ACC CCA CAA CCA CA-3' **P2** 5'-CTG GGC CTT GAA AAA GCA TA -3'
CCR2	60	350	**P1** 5'-TTT GAT CCT GCC CCT ACT TG-3' **P2** 5'-TTT TGG CAA TGT GCT TTC TG-3'
CXCR2	60	197	**P1** 5'-AGT TCT GAC CCG CCC TTT AC-3' **P2** 5'-GCC AGG TTC AGC AGG TAG AC-3'
CXCR4	60	501	**P1** 5'-GCC ATG GCT GAC TGG TAC TT-3' **P2** 5'-GAA GGA ATC GAT GCT GAT CC-3'
ADHESION MOLECULES			
ICAM-1	60	600	**P1** 5'-TTC TGC CAC CAT CAC TGT GT-3' **P2** 5'-TTC TCC ATC TCC AGG GTC TG-3'
VCAM-1	60	451	**P1** 5'-GAC CTG TCA GCG AAG GAA AC-3' **P2** 5'-TGA GCA GGT CAG GTT CAC AG-3'

Table 3.3. Rat primer sequences and product sizes.

3.8.3. Results and Discussion

In the current study the expression of SOCS, cytokines, leukocyte trafficking determinants in a rat model of acute experimental cardiac rejection was assessed. In the model chosen for analysis, for allogeneic grafts Lewis rats serve as recipients for Wistar Furth cardiac transplants (W→L); for syngeneic grafts Lewis rats are used as both donors and recipients (L→L). The combination of Wistar Furth to Lewis is fully mismatched for both MHC class I and class II antigens and thus serves as a model of acute cardiac transplant rejection: cardiac allografts reproducibly undergo irreversible rejection 7-10 days post-transplantation. An animal from each group was sacrificed at days 1, 3, 5, and 7 post-transplantation and both the native (N) and donor (T) heart retrieved, i.e. for each time point post-transplantation 4 hearts were analysed (W→L, N and T; L→L, N and T).

It is important to note that, since intact hearts were used for total RNA extraction, mRNA levels of the species examined reflect *all* cells present in the organ, both resident and infiltrating. In agreement with the current paradigm of acute transplant rejection, the upregulation of immune mediators in the present study was almost exclusively restricted to allogeneic graft tissue. Spleen and lymph nodes were not harvested from the animals, but it has been confirmed in similar models that the temporal expression of immune mediators in these organs is similar to those in acutely rejecting allografts. mRNA levels for many of the key determinants in this process were elevated one day following surgery and most remained above control levels throughout the time course studied, confirming that the immune response is rapid and sustained in this scenario.

Immunohistology

Cryostat sections from native and transplanted hearts were stained for the common leukocyte antigen (CLA, CD45 – a universal marker present on all leukocytes) and the macrophage marker ED1 at all time points. In common with previous reports (109, 133) the current model of unmodified cardiac rejection was characterised by progressive mononuclear cell infiltration. The number of CLA positive and ED1 positive cells in transplanted hearts, both syngeneic and allogeneic, was elevated from day 1 as compared with the native controls – most probably as a consequence of post-operative ischaemia-reperfusion. By day 3 however, marked infiltration of the acutely rejecting mismatched heart by ED1+ cells was found, with the numbers of these cells in these organs, as compared with native hearts and syngeneic controls, remaining elevated over the time course studied (Fig. 3.19a-g). Likewise, pronounced infiltration of allogeneic organs by CLA positive cells, as compared to the matched controls, was apparent from day 5 onwards (Fig. 3.20a-d).

These results confirm that in the current model immune cell infiltration of the rejecting organ is rapid and sustained. Thus having established, in agreement with previous studies, that immunological rejection of allograft tissue occurred throughout the post-transplantation time course, further analyses of cardiac tissue proceeded as detailed below.

Day	Rat	ED1 Staining	
		Native	Transplant
Day 1	L→ L	1+	3+
	WF→ L	1+	3+
Day 3	L→ L	1+	3+
	WF→ L	1+	4+
Day 5	L→ L	1+	2+
	WF→ L	1+	4+
Day 7	L→ L	1+	3+
	WF→ L	1+	4+

Key (approx. cell no.)	
0-200	1+
200-400	2+
400-800	3+
>800	4+

Table 3.4 Graft tissue graded for positivity following IHC staining with anti-ED1.

Day	Rat	CLA Staining	
		Native	Transplant
Day 1	L→ L	1+	2+
	WF→ L	1+	2+
Day 3	L→ L	1+	3+
	WF→ L	1+	3+
Day 5	L→ L	1+	2+
	WF→ L	1+	4+
Day 7	L→ L	1+	2+
	WF→ L	1+	3+

Key (approx. cell no.)	
0-200	1+
200-400	2+
400-800	3+
>800	4+

Table 3.5 Graft tissue graded for positivity following IHC staining with anti-CD 45.

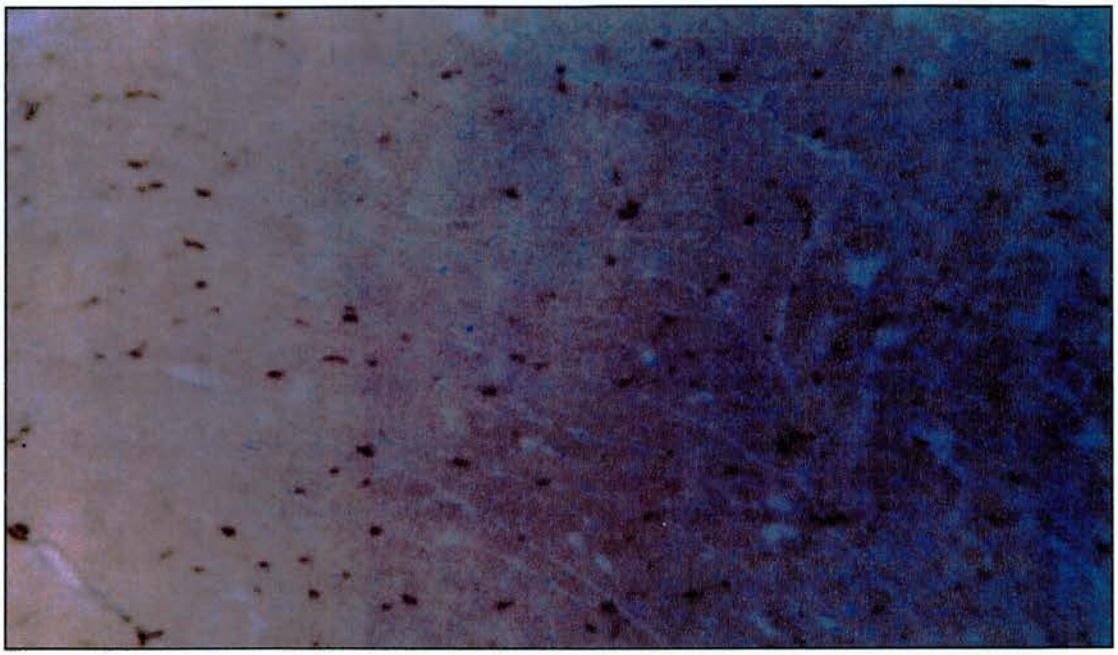


Figure 3.19a ED1 staining in the native heart at day 1 following allogeneic transplant

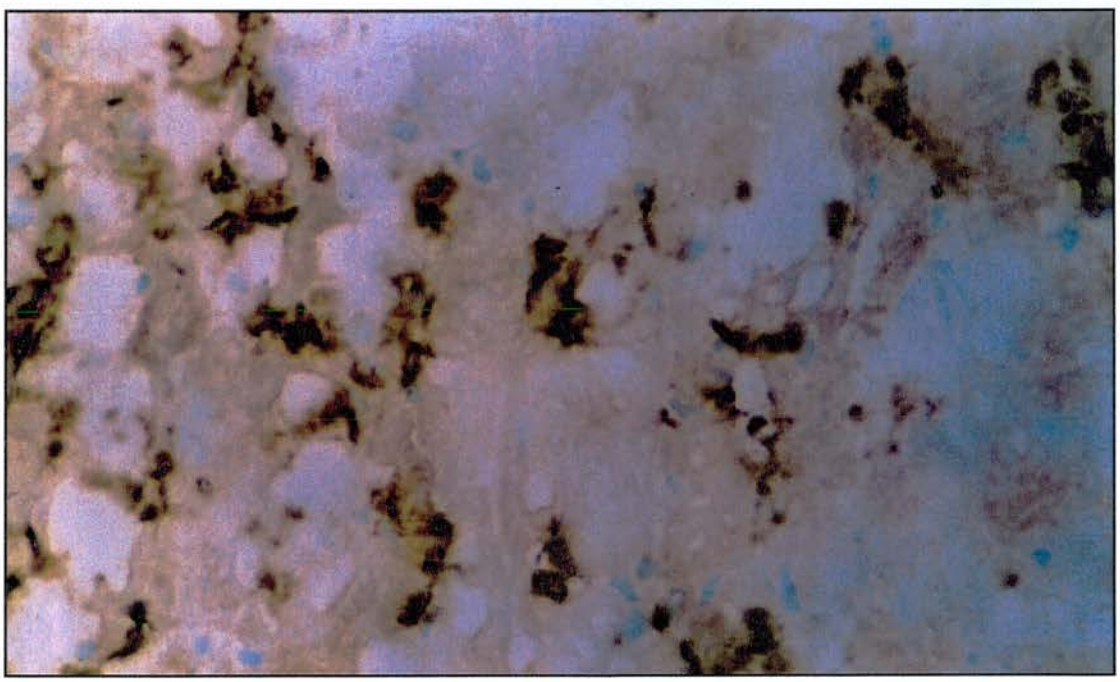


Figure 3.19b ED1 staining at day 1 in allogeneic heart

Sinificant infiltration of ED1 positive cells into the allogeneic organ occurs at day 1, as compared with native heart.

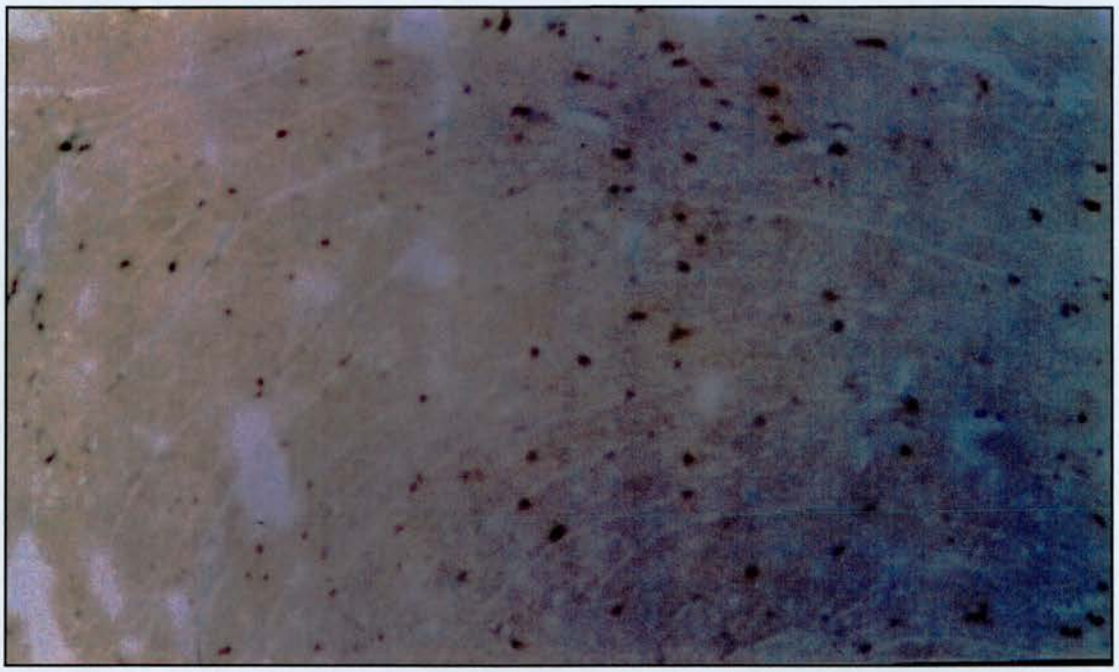


Figure 3.19c ED1 staining in the native heart at day 5 following allogeneic transplant

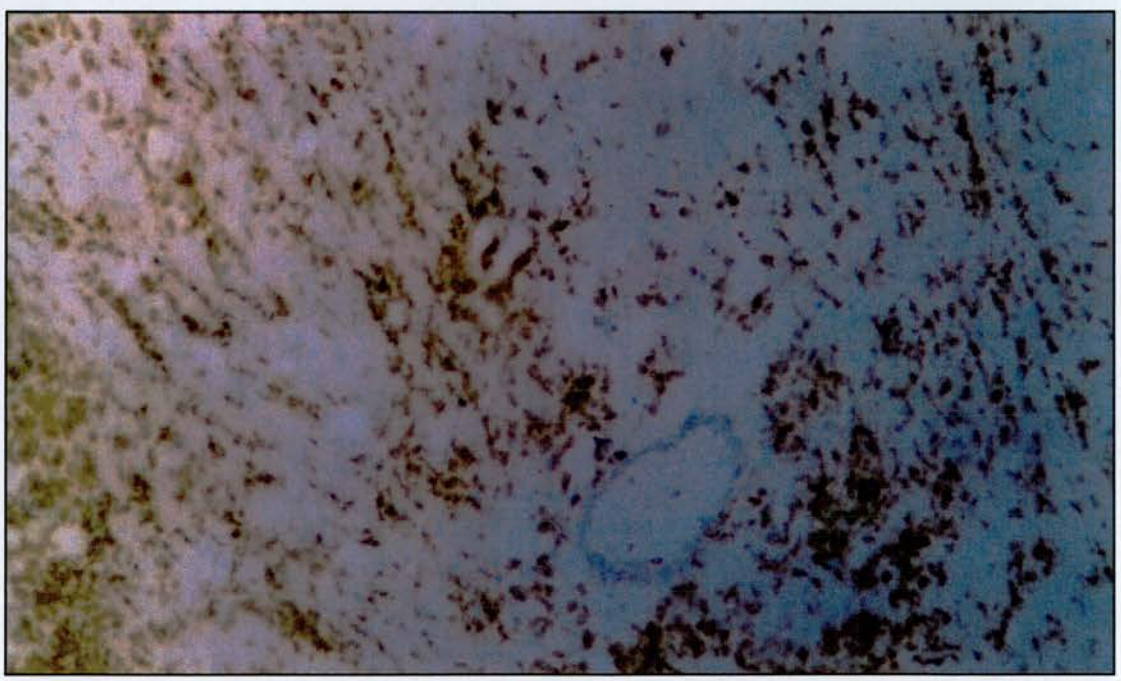


Figure 3.19d ED1 staining at day 5 in allogeneic heart

Marked infiltration of ED1 positive cells into the allogeneic organ occurs at day 5, as compared with native heart.

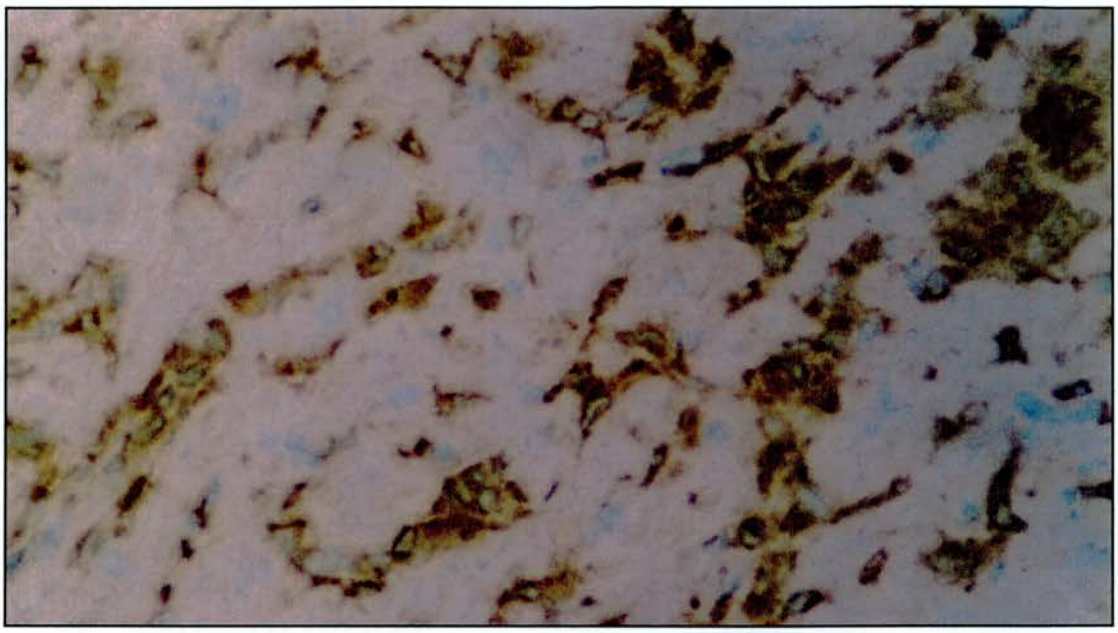


Figure 3.19e ED1 staining at day 5 in the syngeneic heart.

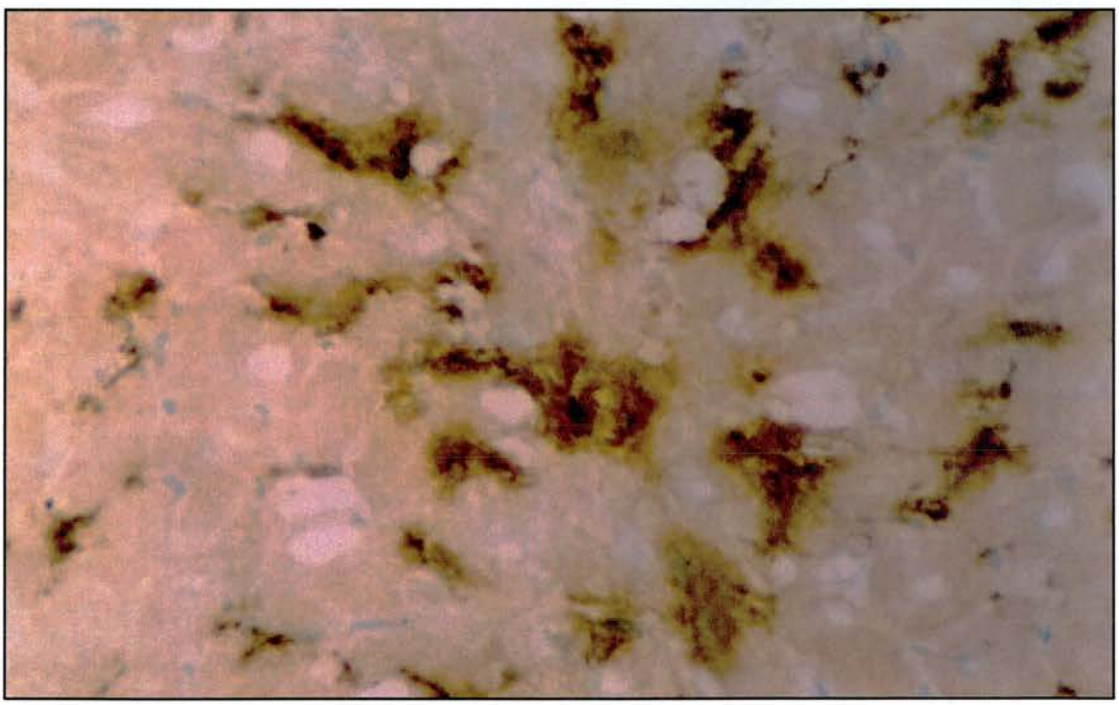


Figure 3.19f ED1 staining at day 5 in allogeneic heart

Marked infiltration of ED1 positive cells into the allogeneic organ occurs at day 5, as compared with syngeneic heart.

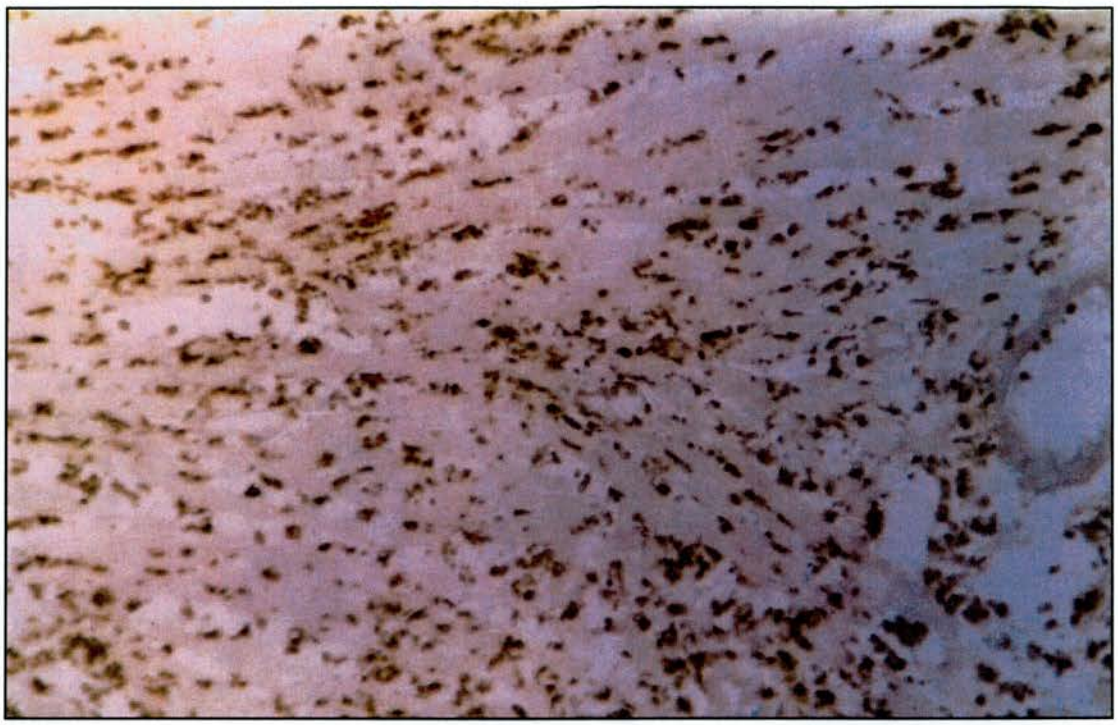


Figure 3.19g ED1 staining in the allogeneic heart at day 7.

Ongoing infiltration by ED1 positive cells occurs at day 7 in the allogenic organ

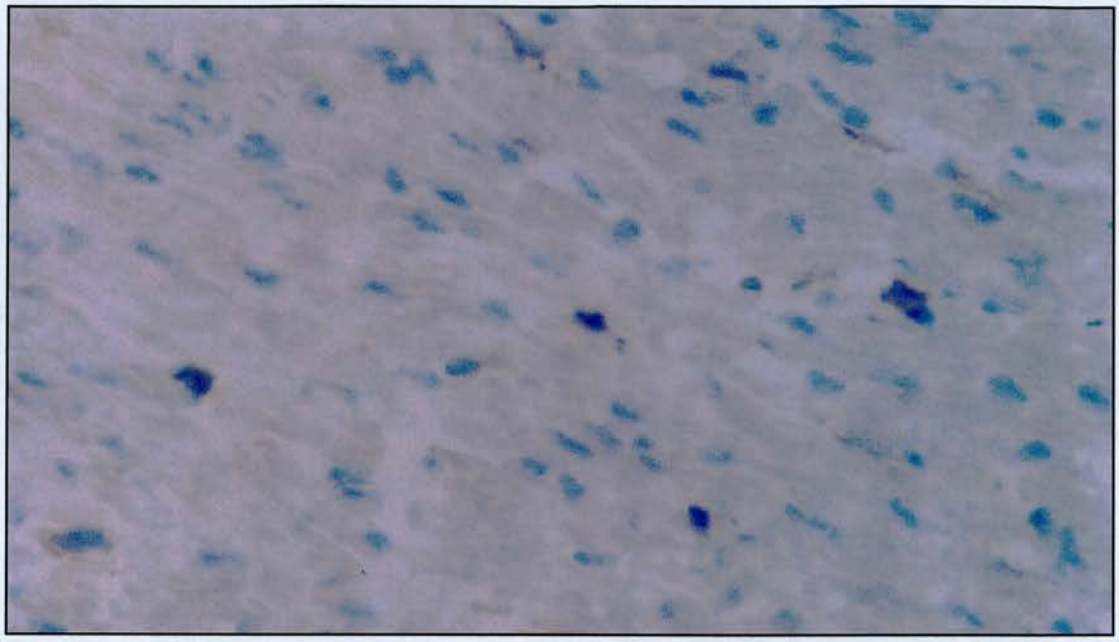


Figure 3.20a CD 45 staining in the native heart at day 1 following allogeneic transplant

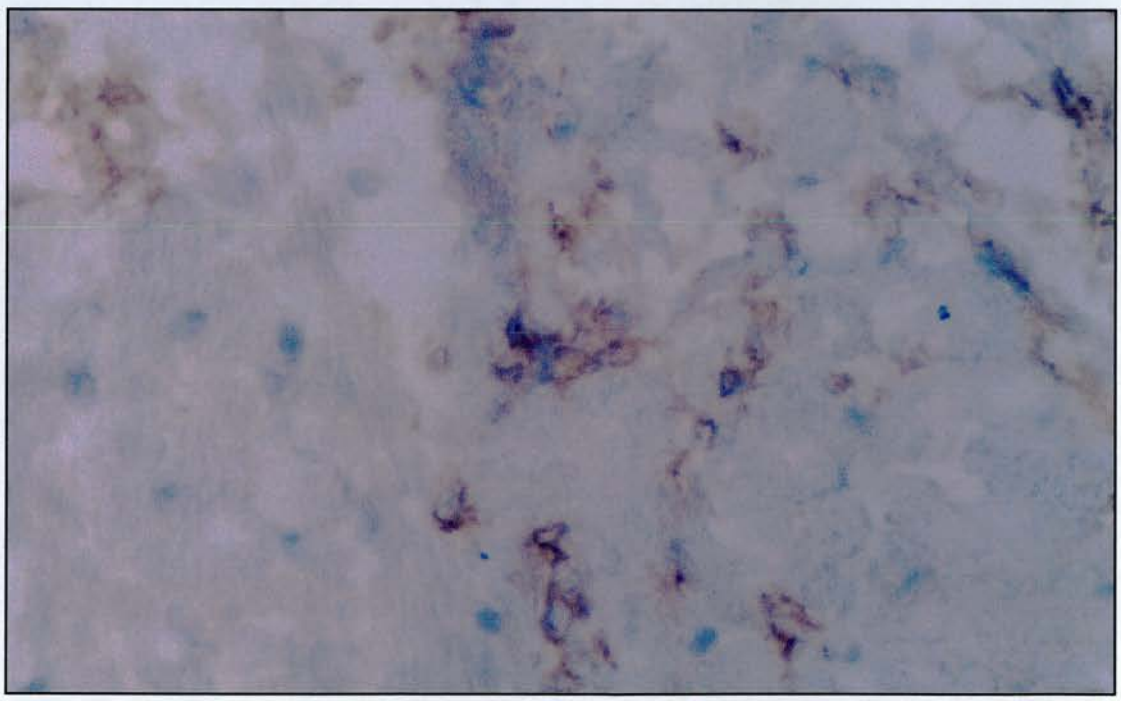


Figure 3.20b CD45 staining at day 1 in allogeneic heart

Sinificant infiltration of CD 45 positive cells into the allogeneic organ occurs at day 1, as compared with native heart

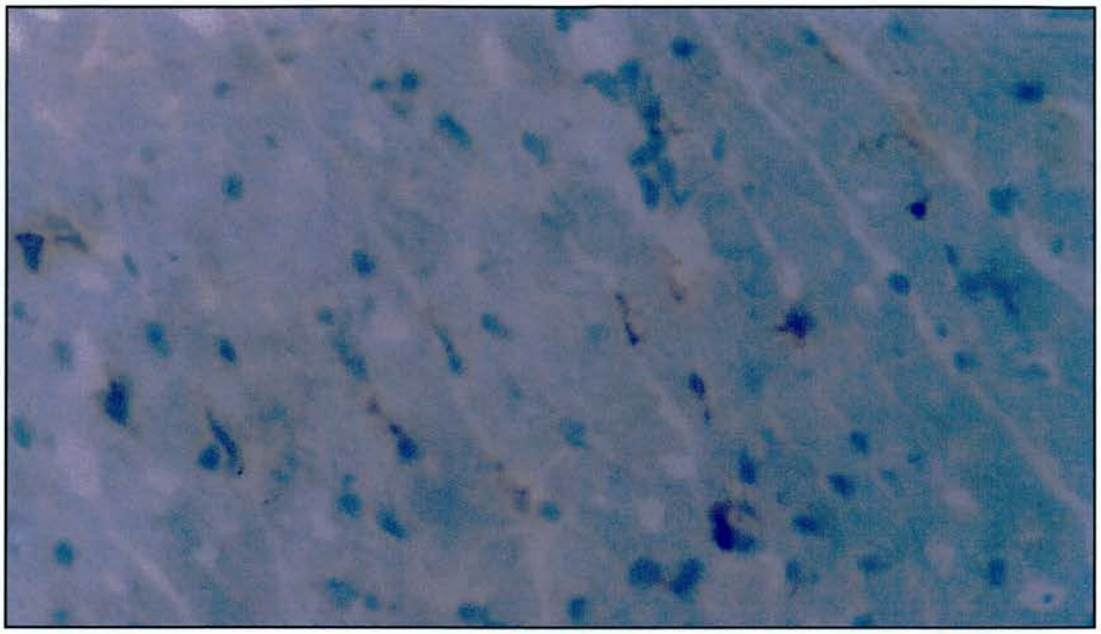


Figure 3.20c CD 45 staining in syngeneic organ at day 5 following transplant

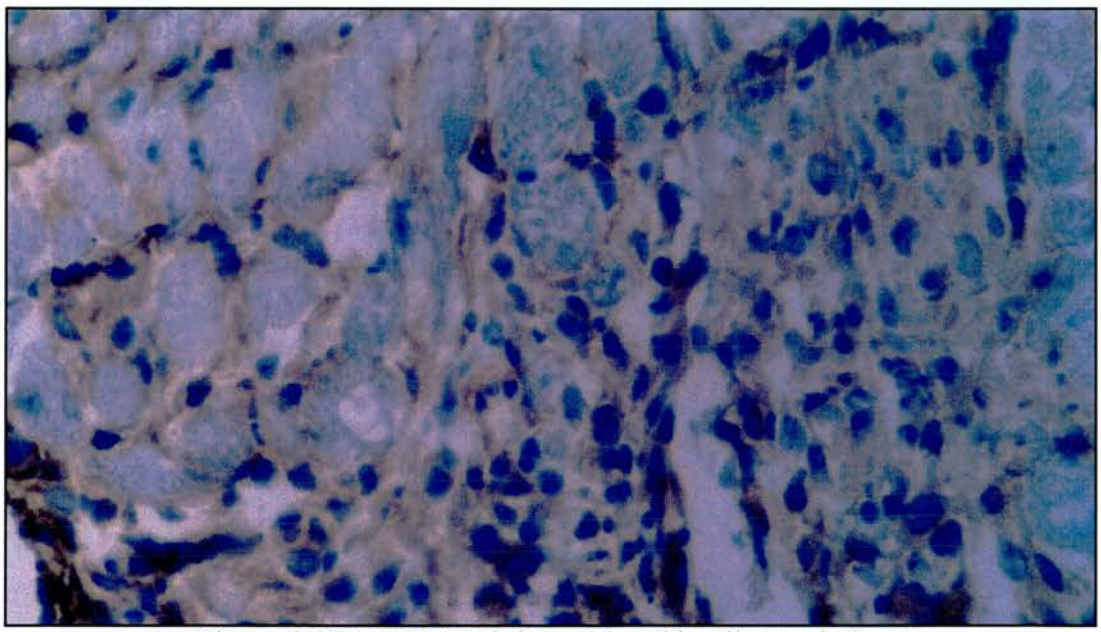


Figure 3.20d CD45 staining at day 5 in allogeneic heart

Pronounced infiltration of CD 45 positive cells into the allogeneic organ occurs at day 5, as compared with syngeneic heart.

SOCS expression

Cytokines are central to the generation of the allogeneic immune response. These mediators are produced by multiple different cell types including T cells, B cells, monocytes/macrophages and antigen presenting cells as well as various non-immune cells including endothelial cells. Cytokines initiate their pleiotropic cellular effects through their interactions with specific receptors on the surface of target cells. Once bound to the surface receptor various intracellular signal transduction pathways are activated and ultimately these lead to the transcription of new genes. The importance of the JAK/STAT signal transduction cascade in linking activation of cytokine receptors to gene transcription has been established. Recently a family of inhibitors of JAK/STAT signalling have been reported, the *s*uppressors *o*f *c*ytokine *s*ignalling (SOCS) family. These are discussed in detail in section 3.6 and their profile in an experimental model of glomerulonephritis is detailed in section 3.7. We sought therefore to examine the expression of the SOCS family members in another model of acute inflammation – acute transplant rejection.

Levels of SOCS-3 mRNA were elevated specifically in allogeneic transplants on days 1-7. No SOCS-3 expression could be detected in native hearts or syngeneic controls (Fig. 3.21). CIS-1 expression occurred in all specimens, but at day 5 levels in the allogeneic graft, as compared to the control tissues were elevated (Fig 3.22). SOCS-1 was weakly expressed in rejecting organ at day 5 only (Fig 3.23). RT-PCR analysis of SOCS-2 revealed constitutive expression but no allogeneic-specific upregulation of this species (Fig 3.24).

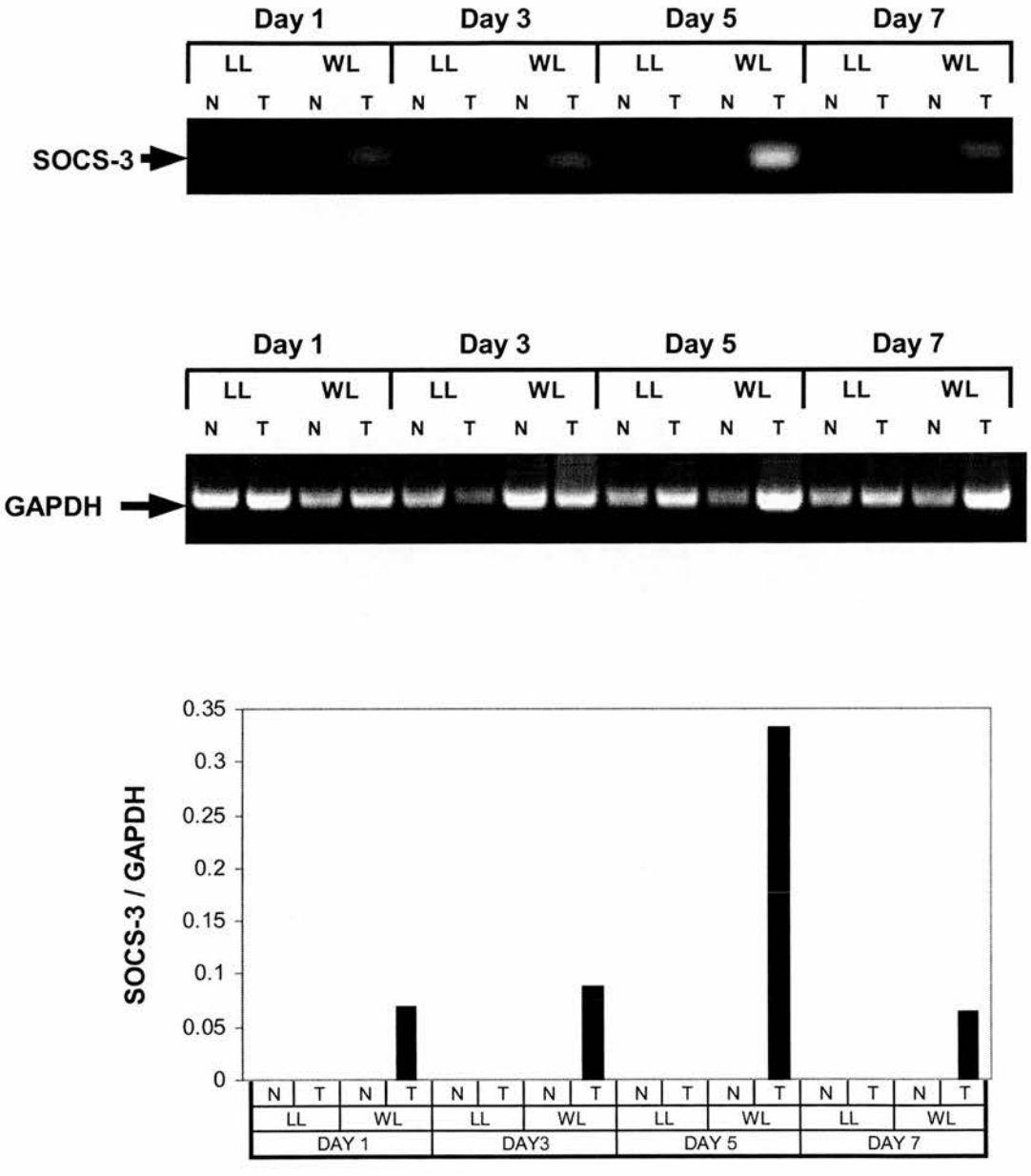


Figure 3.21. SOCS-3 expression in experimental acute cardiac transplant rejection

Levels of SOCS-3 mRNA were elevated specifically in allogeneic transplants on days 1-7. No SOCS-3 expression could be detected in native hearts or syngeneic controls

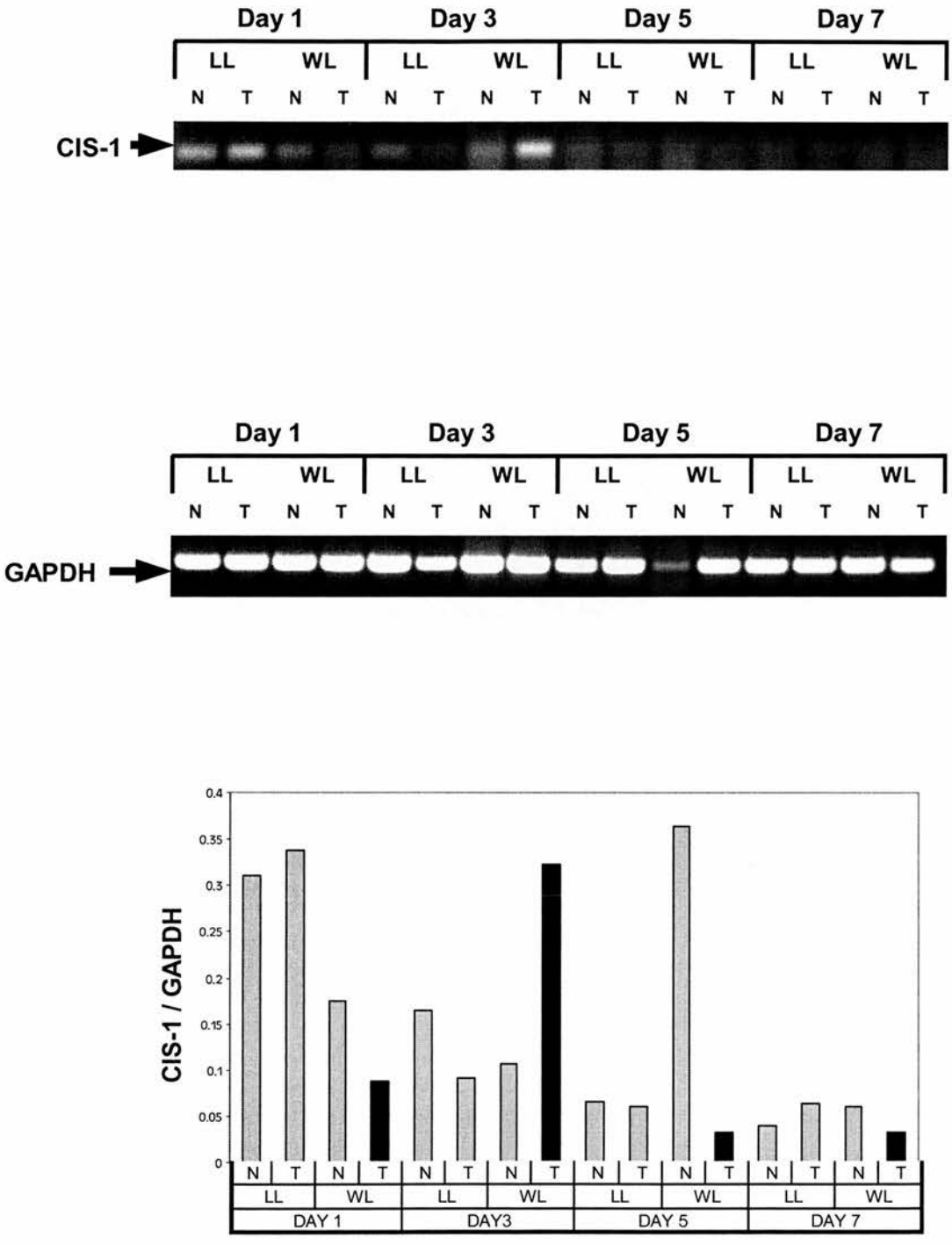


Figure 3.22. CIS-1 expression in experimental acute cardiac transplant rejection

CIS-1 expression occurred in all specimens, but at day 5 levels in the allogeneic graft, as compared to the control tissues were elevated

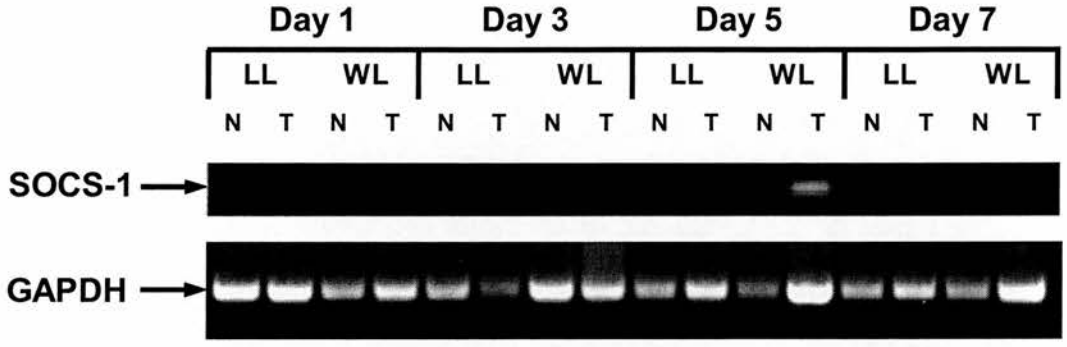


Figure 3.23 SOCS-1 expression in experimental acute cardiac transplant rejection

SOCS-1 expression was detected only in the day 5 allogeneic organ.

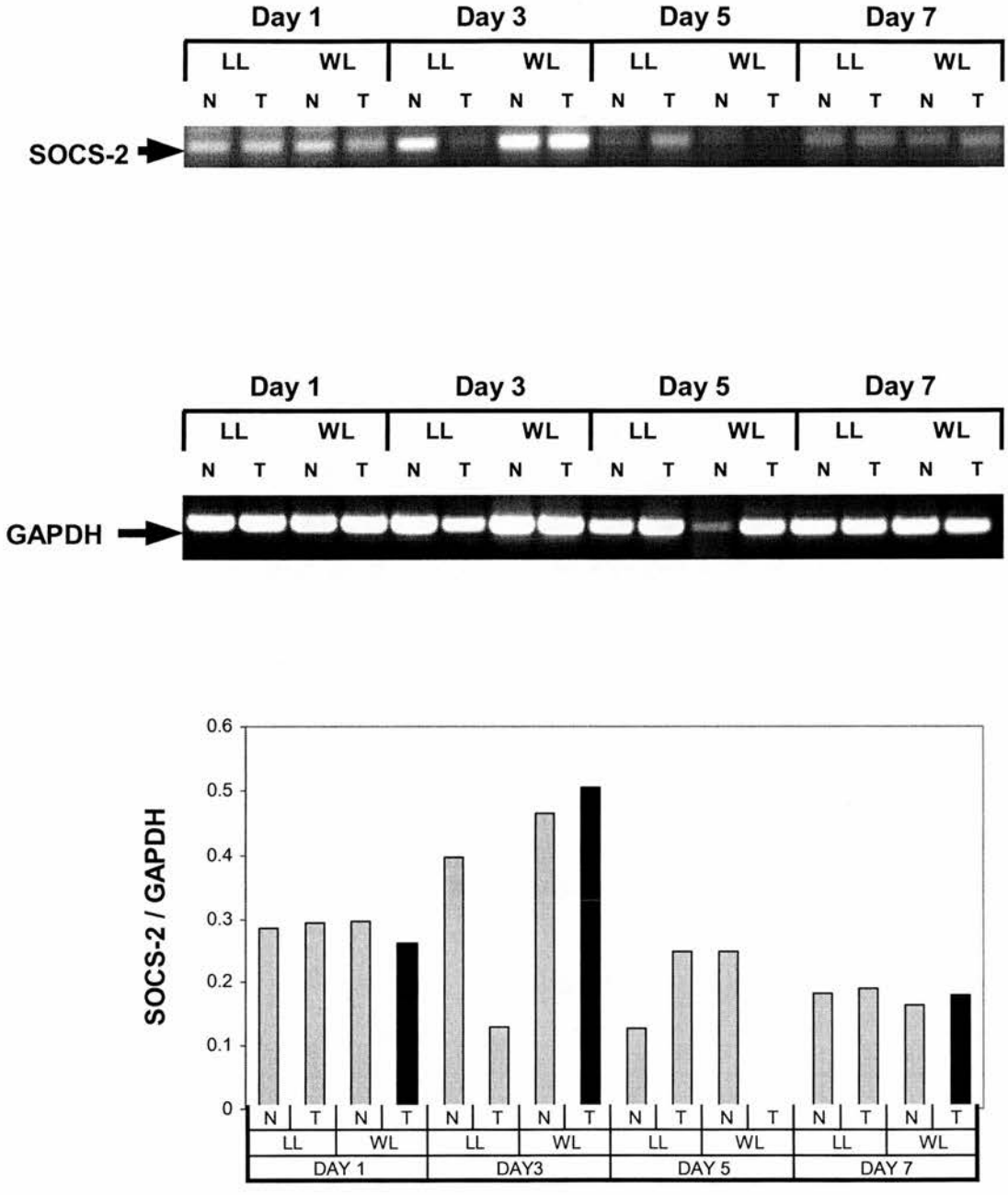
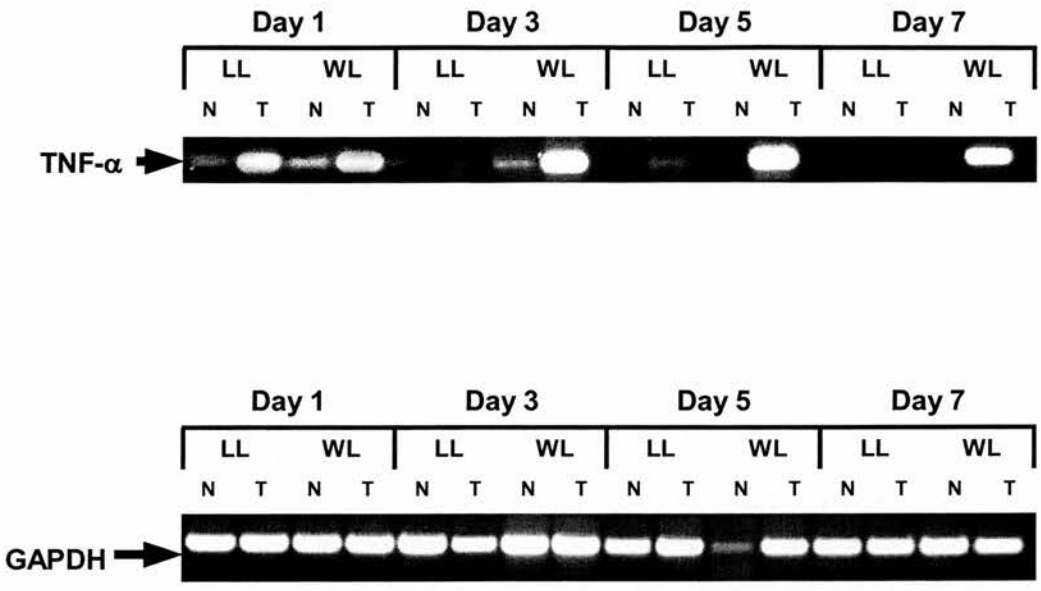


Figure 3.24 SOCS-2 expression in experimental acute cardiac transplant rejection

SOCS-2 expression occurred in all specimens but no allogeneic-specific upregulation of this species was demonstrated.

Cytokine expression

Having confirmed the presence of SOCS in this model, we then sought to establish the simultaneous presence of a variety of representative cytokines known to be important in the rejection process. mRNA for the cytokine TNF-α was present in all specimens at day 1, but more pronounced in the transplanted hearts (allogeneic and syngeneic). After day 1 however mRNA for this species declined in control tissues while levels in the mismatched grafts continued to increase and remained elevated compared to the control at all time points (Fig 3.25). Similarly, IL-1β was detected in native hearts, syngeneic transplants and allogeneic transplants at day 1. On days 3, 5 and 7, however, expression levels for this cytokine rose markedly in mismatched transplants when compared to native hearts and syngeneic controls. (Fig 3.26) The universal expression of these mRNA species on day 1 was most probably a post-operative phenomenon and, in particular, increased expression of TNF-α in the transplanted hearts on day 1, and its subsequent decline in syngeneic tissue, is most likely secondary to ischaemia-reperfusion injury occurring specifically in the transplanted hearts. The expression of IL-2 and IFN-γ was identified only in allogeneic transplants. mRNA for IL-2 was apparent from day 3 onwards while that for IFN-γ was detectable at days 5 and 7 only (Fig. 3.27, Fig. 3.28). In contrast, expression of the Th2 cytokine IL-4 was not detected in native hearts, isograft controls or allogeneic hearts over the time course studied, while IL-10 mRNA was apparent only in allogeneic organs from day 3 onwards (Fig. 3.29).



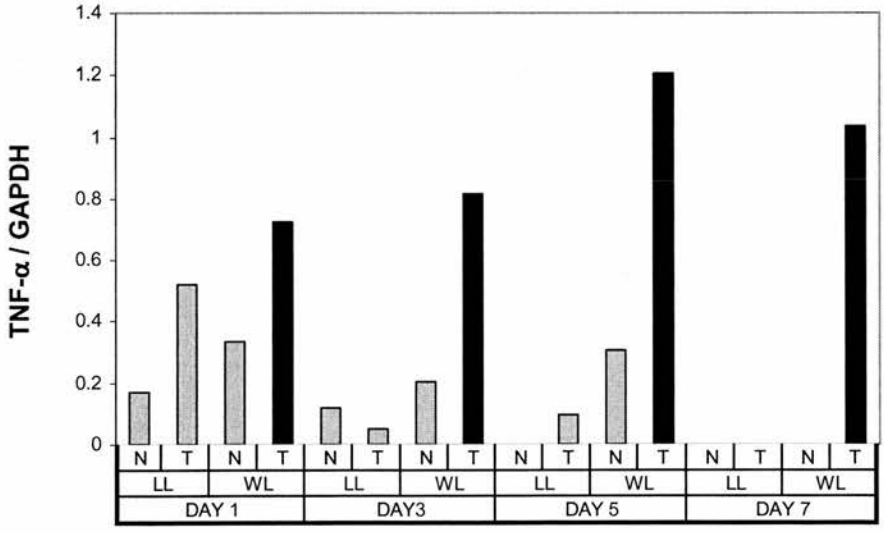


Figure 3.25 TNF-α expression in experimental acute cardiac transplant rejection

mRNA for the cytokine TNF-α was present in all specimens at day 1, but more pronounced in the transplanted hearts (allogeneic and syngeneic). After day 1 however mRNA for this species declined in control tissues while levels in the mismatched grafts continued to increase and remained elevated compared to the control at all time points

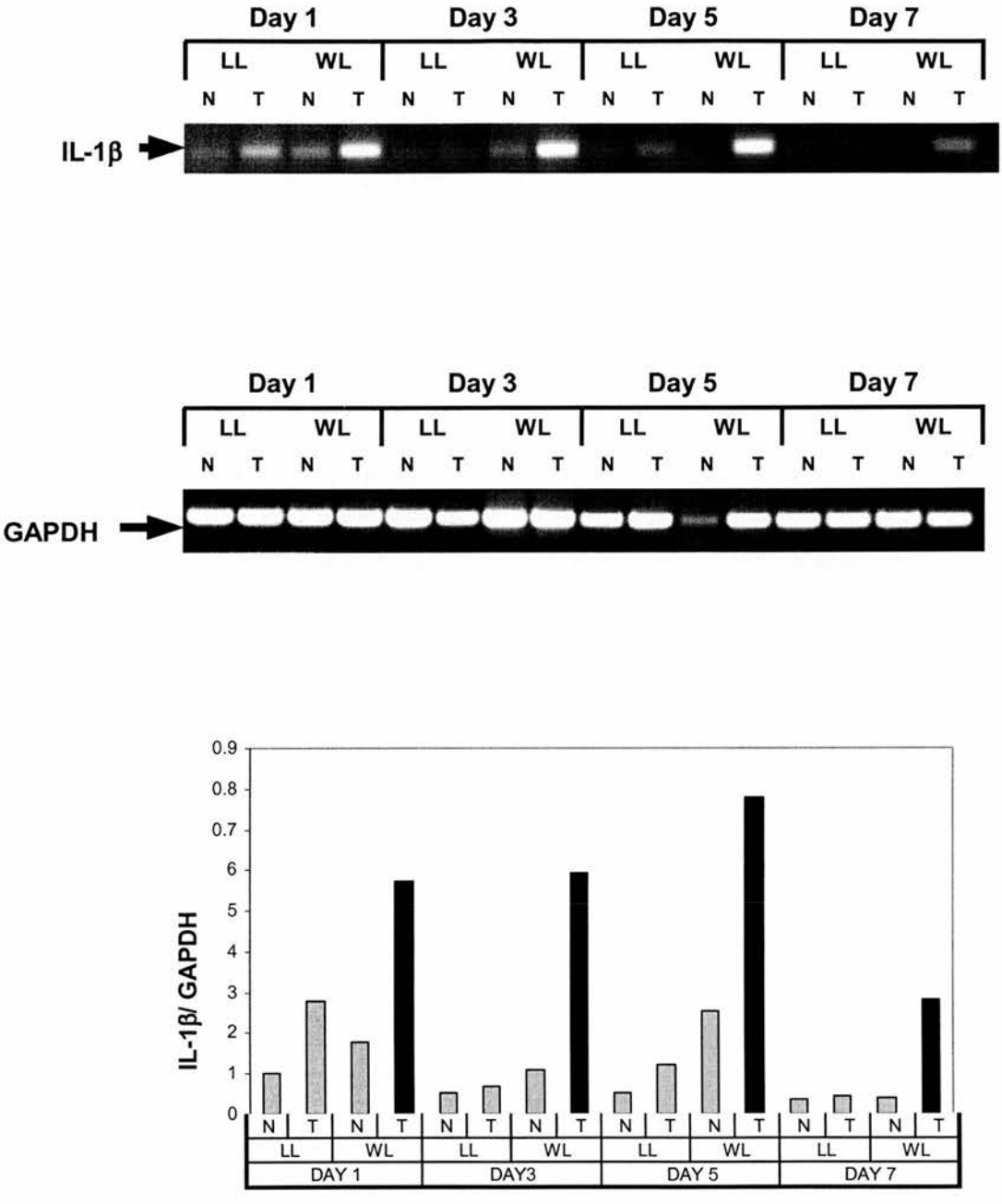


Figure 3.26 IL-1β expression in experimental acute cardiac transplant rejection

IL-1β was detected in native hearts, syngeneic transplants and allogeneic transplants at day 1. On days 3, 5 and 7, however, expression levels for this cytokine rose markedly in mismatched transplants when compared to native hearts and syngeneic controls

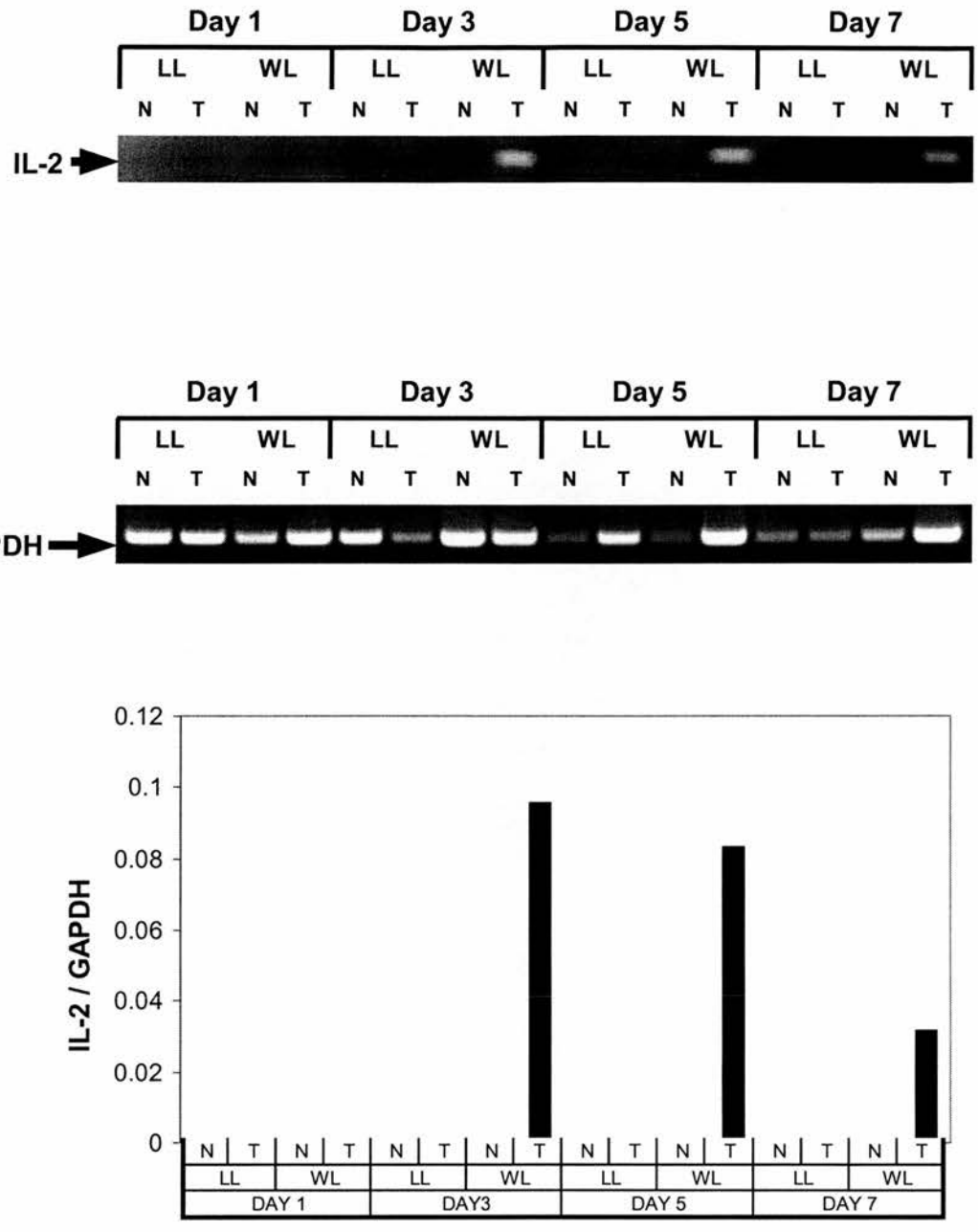


Figure 3.27 IL-2 expression in experimental acute cardiac transplant rejection

The expression of IL-2 was identified only in allogeneic transplants. from day 3 onwards.

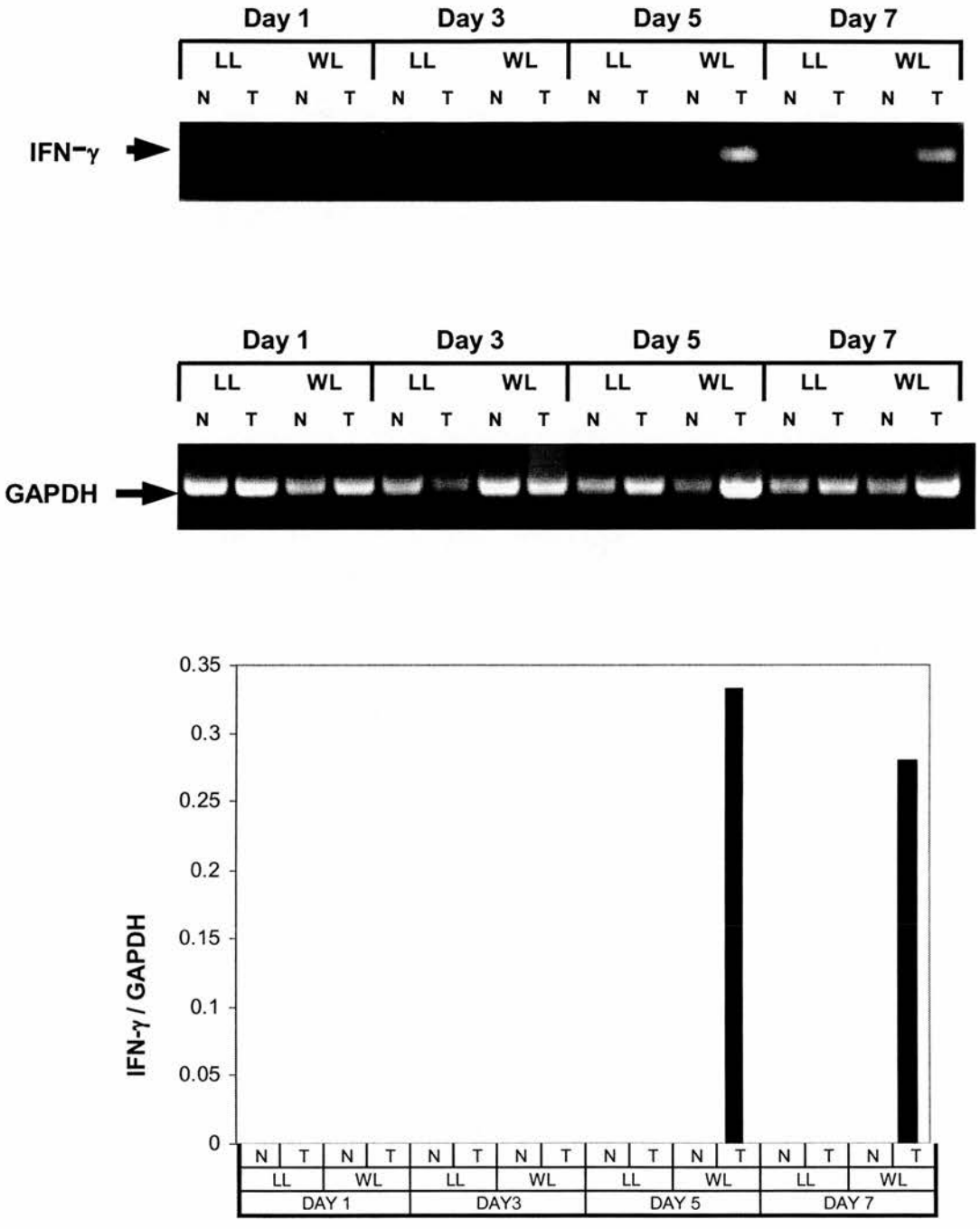


Figure 3.28 IFN-γ expression in experimental acute cardiac transplant rejection

The expression of IFN-γ was identified only in allogeneic transplants at days 5 and 7

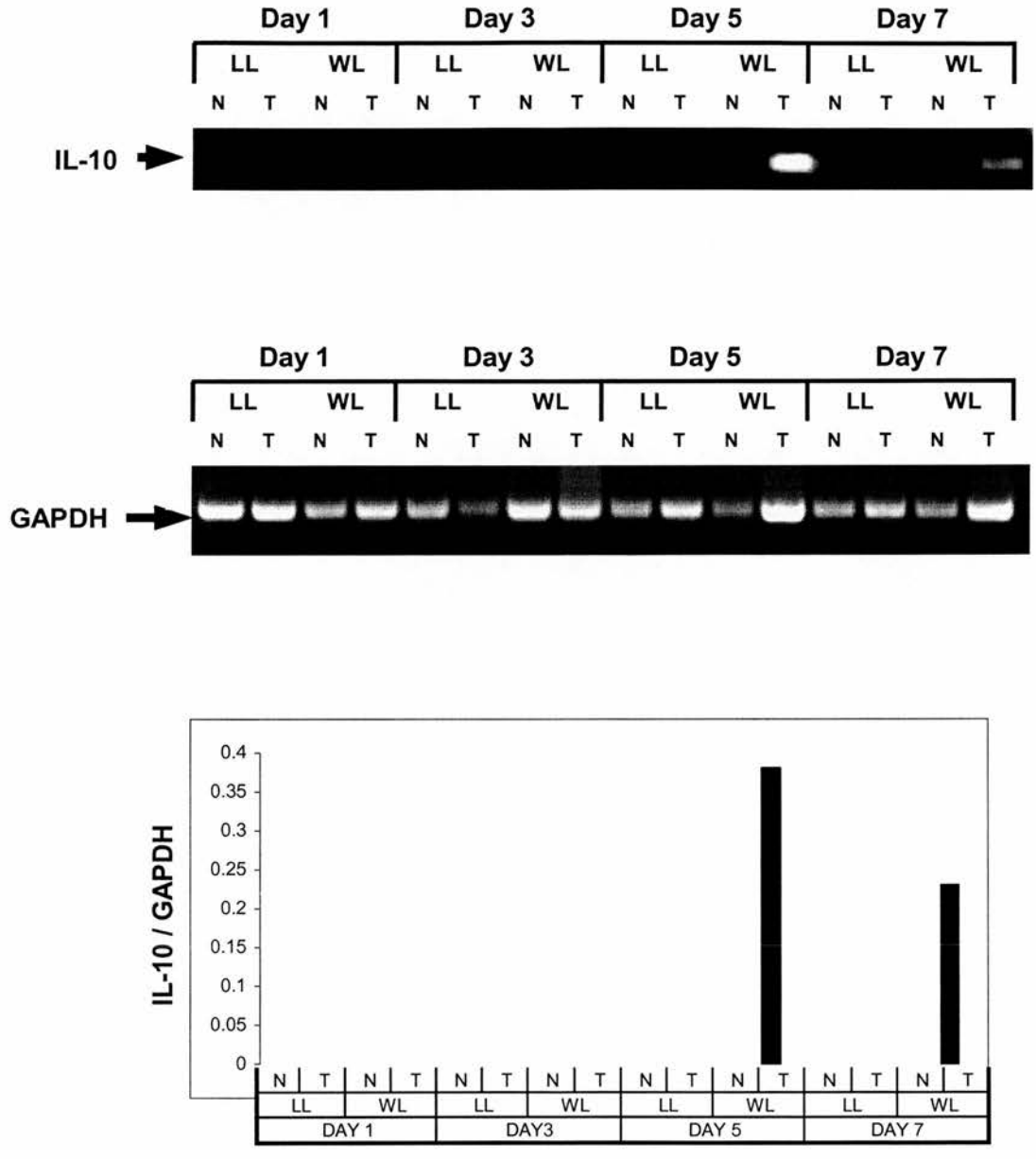


Figure 3.29 IL-10 expression in experimental acute cardiac transplant rejection

The expression of IL-10 was identified only in allogeneic transplants at days 5 and 7.

T cells and their products are critical in the acute rejection event. Two major subsets of T cells, the $CD8^+$ cytotoxic T cells and $CD4^+$ T helper (Th) cells recognise processed antigen on MHC class I and class II, respectively. Subsets of Th cells, namely Th1 and Th2, which exhibit characteristic cytokine production profiles are central to the immune response (section 1.3.5). Th1 cells exert broadly pro-inflammatory actions via their secretion of IL-2, TNF-α and IFN-γ, while Th2 cytokines (principally IL-4, IL-6, IL-10 and IL-13) exert mainly anti-inflammatory effects via their suppression of macrophage and Th1 cell activation. IL-2 is a powerful autocrine T cell growth factor, activating T cells, stimulating their growth and giving rise to clonal expansion. In addition, this cytokine facilitates the effector function of cytotoxic T cells. TNF-α and IFN-γ, secreted both by Th1 cells and macrophages, exert activating effects on monocytes and resident cells. These cytokines induce class I and class II antigen expression, modulate adhesion molecule expression and stimulate the release of further cytokines, chemokines and growth factors from monocytes, T cells and endothelial cells. IL-1β production by antigen-activated macrophages further enhances the immune response by stimulating growth factor and chemokine production by resident cells thus promoting further infiltration of lymphocytes and macrophages into the allograft. IL-1β also acts in an autocrine fashion to induce its own synthesis.

The cytokines IL-4 and IL-10 are produced mainly by Th2 cells. These cytokines have multifunctional roles, the most important of which is the modulation of macrophage function (3). Th2 cytokines have been shown to inhibit macrophage release of reactive oxygen species (ROS), proinflammatory cytokines and chemokines. In addition, these anti-inflammatory mediators down-regulate leukocyte

growth factors, including granulocyte macrophage colony-stimulating factor (GM-CSF) and granulocyte colony stimulating stimulating factor (G-CSF). All of these effects result in a marked attenuation of the inflammatory process. The role of IL-10, however, is particularly complex in that its effects are not uniformly immunosuppressive or anti-inflammatory: IL-10 augments IL-2-driven proliferation of cytotoxic T cells and enhances IL-2 induced cytotoxicity by these cells. In addition this cytokine stimulates antibody dependent cellular cytotoxicity. Thus, depending on the nature of the APC and the local microenvironment, IL-10 may exert anti- or pro-inflammatory effects. The presence of IL-10, and its related cytokines, is well-documented in experimental transplant models treated with immunosuppression or manipulated to induce tolerance where immune deviation from a Th1 to a Th2 phenotype occurs. Conflicting reports exist as to the expression of this cytokine in models such as the one examined. Our observation that IL-10, but not IL-4, is expressed in the allogeneic transplants may indicate that in this scenario IL-10 is behaving as a pro-inflammatory cytokine or indeed raises the possibility that the IL-10 transcripts observed were not derived from Th2 lymphocytes, but from cells of a monocyte lineage (134).

The observations in the current study that expression of pro-inflammatory cytokines is restricted to mismatched allografts confirms that in acute cardiac rejection, the inflammatory response is largely confined to the graft tissue and implicates these cytokines in the promotion and amplification of this response. These results are in broad agreement with those of other investigators using other experimental acute transplant rejection models including murine heterotopic cardiac transplantation models, alternative rat heterotopic cardiac transplantation models exchanged over

different MHC mismatches and, indeed, acutely rejecting renal allografts in rats (117, 135, 136). Analyses in these experimental settings have demonstrated that acute rejection is dominated by intragraft production of pro-inflammatory cytokines (IL-2, IFN-γ, TNF-α, IL-1β) while graft acceptance, in general, is associated with diminished type 1 and enhanced type 2 cytokine production (137).

The complex actions of the cytokine TGF-β are essential in a wide range of pathophysiological processes. Constitutive expression is essential for the maintenance of normal immune and tissue function; TGF-β "knock-out" mice demonstrate immune dysregulation, multiorgan inflammation and early death (53). TGF-β is upregulated in many diseases particularly autoimmune disorders where it appears to exert anti-inflammatory actions (3). In this model, TGF-β expression was detected only in allogenic transplants from day 3 onwards and remained elevated over the time course studied (Fig. 3.30). Connective tissue growth factor (CTGF) is a downstream mediator of TGF-β action on connective tissue cells, where it stimulates cell proliferation and synthesis of ECM (138). Studies have demonstrated that CTGF is coordinately expressed with TGF-β in every fibrotic disorder examined to date. In the current model CTGF, although constitutive in all specimens, was not specifically upregulated in mismatched transplants (Fig 3.31). The upregulation of TGF-β during cardiac allograft rejection most likely represents endogenous immunosuppression by infiltrating lymphocytes. TGF-β also mediates potent fibrogenic effects which can be propagated, in part, by the actions of CTGF.

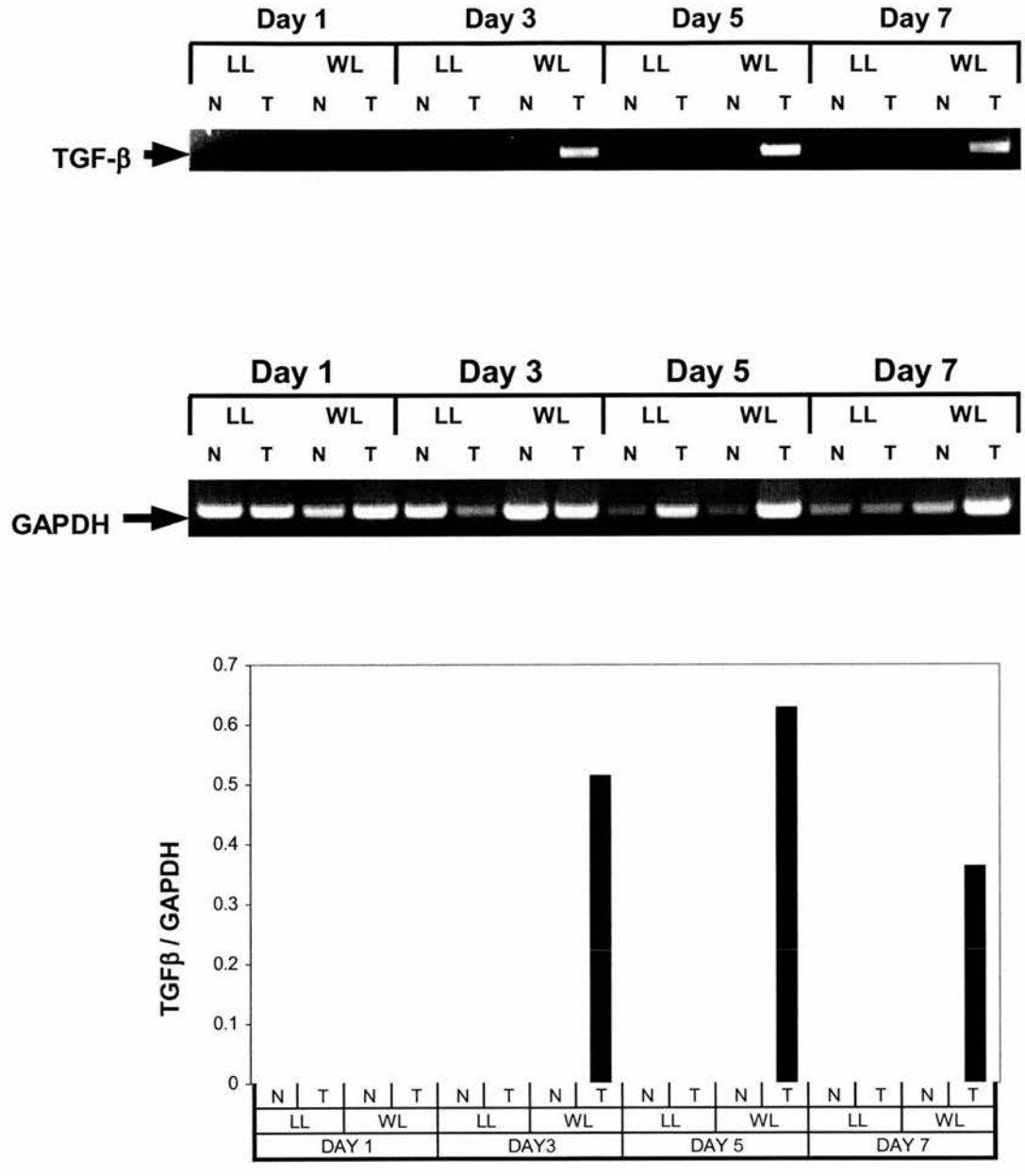


Figure 3.30. TGF-β expression in experimental acute cardiac transplant rejection

TGF-β expression was detected only in allogeneic transplants from day 3 onwards and remained elevated over the time course studied.

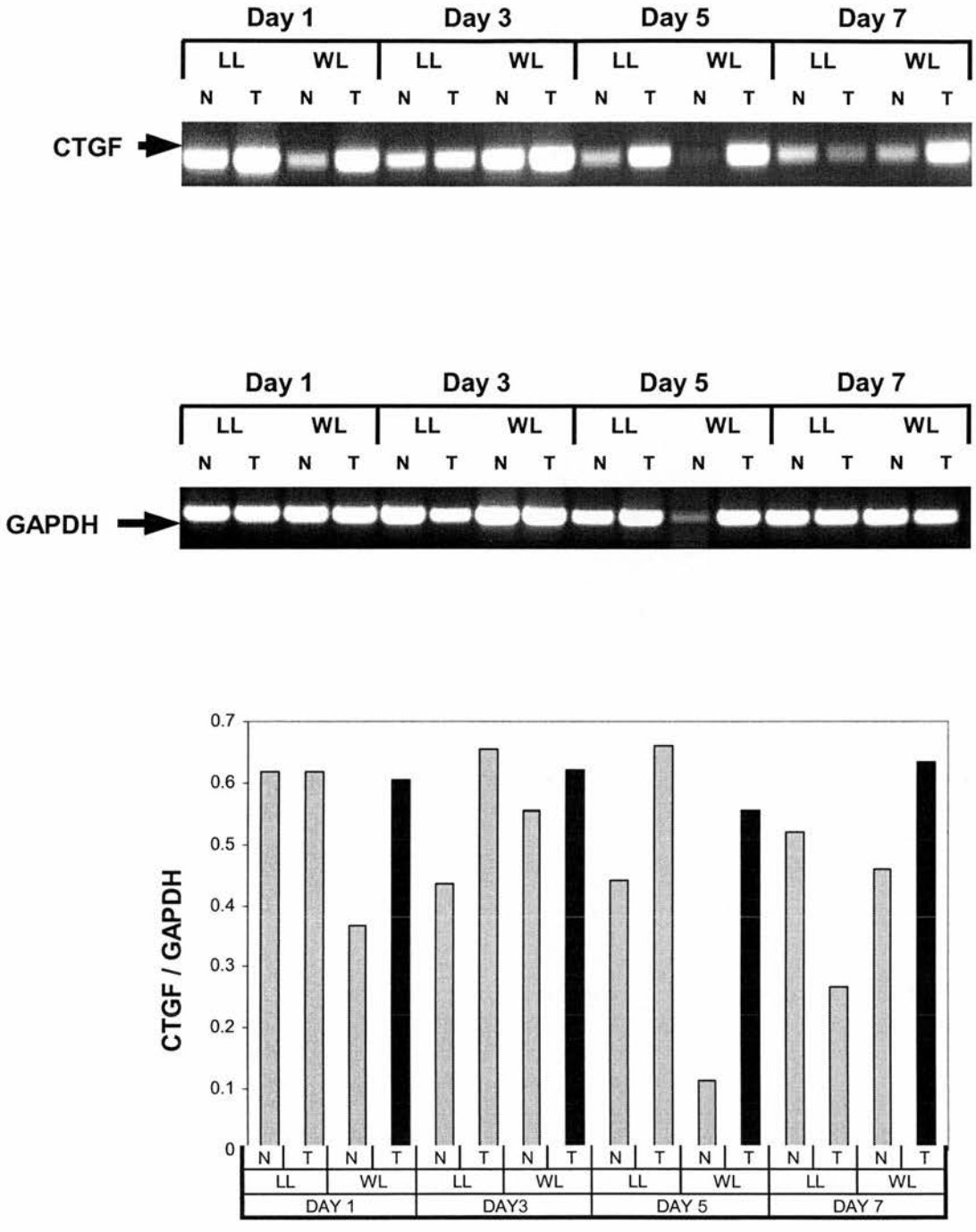


Figure 3.31 CTGF expression in experimental acute cardiac transplant rejection

CTGF, was constitutive in all specimens but not specifically upregulated in mismatched transplants

In acute allograft rejection, and certainly over the time course studied here, fibrosis is not a prominent feature (confirmed by histology) and therefore the failure to demonstrate increased mRNA for CTGF in allogeneic transplants is not unexpected. It would be interesting however to profile the expression of this mediator in an experimental model of chronic cardiac rejection or indeed in human chronic cardiac rejection in which fibrosis is a more prominent feature.

SOCS, cytokines and immune cell infiltration

The current findings that SOCS-2 and CIS-1 are constitutively expressed are in broad agreement with previous studies which have reported basal SOCS-2 and CIS-1 expression in murine heart. SOCS-3 expression in bone marrow is increased by a wide range of pro-inflammatory cytokines including IL-1β, IL-2, TNF-α and IFN-γ, so it is not unexpected that increased levels of this mRNA species were detected in the mismatched transplants where the ambient levels of these cytokines are elevated as a consequence of the activation of the inflammatory response. The mechanisms by which SOCS-3 inhibits the JAK/STAT cascade are currently unknown. Although SOCS-3 interacts with JAK-2 *in vitro,* it suppresses JAK activity only weakly, despite a pronounced inhibition of cytokine bioactivity (80, 83). The cytokines upregulated in the current study signal through multiple JAKS and STATs (or STAT-like factors), any number of which are potential targets for SOCS-3 activity.

In contrast with the other SOCS family members, SOCS-1 is only faintly detected in allogeneic tissue at day 5. Previous investigators have shown that basal SOCS-1 expression was not detected in murine heart and that SOCS-1 expression in bone marrow increases in response to IFN-γ but not in response to other pro-inflammatory

cytokines such as IL-2, TNF-α or IL-1β (60). In murine cell lines SOCS-1 is an interferon-γ inducible gene while overexpression of SOCS-1 in these cell lines confers IFN-γ resistance (88). Furthermore, SOCS-1 knock-out mice exhibit excessive responses typical of those induced by IFN-γ (hyper-responsiveness to viral infection and enhanced IFN-γ-dependent macrophage function) (139). This was prevented by the administration of anti-IFN-γ antibodies and did not occur in SOCS $1^{-/-}$ mice also lacking the IFN-γ gene. On the basis of these observations it has been speculated that SOCS-1 is a key modulator of IFN-γ actions and may allow the protective effects of this cytokine to occur without the risk of associated pathological responses. In the context of the current study IFN-γ expression in allogeneic organs, like SOCS-1 expression, does not occur until day 5. It has been previously shown that the upregulation of SOCS-1 in murine liver in response to IL-6 was rapid and declined to basal levels within 4 hours. SOCS-1 contains a potential PEST sequence; pest sequence containing proteins are predisposed to enhanced ubiquitination and degradation. Indeed difficulties encountered by other investigators in reproducibly expressing SOCS-1 to a moderate level in mammalian cells *in vitro* were overcome by the addition of a GST-tag to the NH2 terminal of SOCS-1, hence conferring resistance to degradation (82). These observations may explain the presence of SOCS-1 in only the day 5 allogeneic organ and why, even on this day, expression was only barely apparent.

The upregulation of CIS-1, as compared to its expression in native hearts and syngeneic control, occurred only at day 3. Negative regulation of the JAK/STAT pathway by CIS-1, at least in part, occur via its inhibition of STAT 5 proteins in a

number of *in vitro* models. (73). Activation and recruitment of STAT 5 occurs in response to multiple cytokines including IL-2, IL-3, IL-5, GM-CSF and erythropoietin (57). In the current study IL-2 expression was only detectible in allogeneic organ at day 5, and the expression of these other known STAT 5 activators was not investigated. Other studies, however, in murine systems have documented increased expression of IL-3, IL-5 and GM-CSF in allogeneic organs (135). The upregulation of CIS-1 at day 3 in the mismatched organ may reflect the presence of such STAT 5-activating cytokines.

SOCS-2, although present in all organs, demonstrated no allogeneic-specific upregulation. While SOCS-2 has been previously been demonstrated to be constitutive in murine heart, expression in bone marrow was increased in response various cytokines including IL-1 and IFN-γ (60). The findings of the present study may reflect both species and tissue differences in the nature of cytokine-induced SOCS-2 upregulation.

The sustained rise of SOCS-3 in allogeneic organs as compared with increases of CIS-1 and SOCS-1 at days 3 and 5 respectively is intriguing. Basal and cytokine-induced SOCS expression exhibits striking tissue- and cell-type specificity (section 3.6). Recent evidence suggests that certain chemokines such as RANTES and MIP-1α may also activate the JAK/STAT pathway and indeed many of the mechanisms of chemokine receptor-chemokine signal transduction are still unknown (140, 141). The upregulation of multiple cytokines has been demonstrated in this model. In addition, by virtue of the fact that whole organ was used for RNA analysis, these results reflect a heterogeneous cell population. We cannot speculate, therefore, on the basis of these

results as to which particular cytokines induce expression of particular SOCS species. Profiling the expression of SOCS in mice in which "knock-out" technology has deleted the gene for a particular cytokine and who subsequently undergo organ transplantation would undoubtedly clarify this. To our knowledge this is the first report of SOCS expression in animal transplant rejection. These findings raise the tantalising possibility that SOCS-3, and indeed the specific cytokines which induce its upregulation, may be important determinants in the process of acute cardiac rejection.

Chemokine/chemokine receptor expression

The leukocyte population infiltrating the allograft is defined both by the nature of the chemokines secreted by, as well the specific chemokine receptors expressed on, the cytokine-activated allograft cells and the infiltrating cells themselves. Given the central role of the nature of this infiltrating leukocyte population in promoting and sustaining the alloreactive immune response, the expression of a variety of representative C-C and C-X-C chemokines and their corresponding receptors was profiled in this model.

While expression of RANTES was constitutive in all specimens, mRNA levels for this chemokine was elevated in allogeneic allografts as compared to controls on days 3, 5 and 7 (Fig 3.32). mRNA for GRO was detected, allbeit at low levels, in control specimens at day 1, while levels in the mismatched transplant at this time-point were markedly elevated. On days 3-7, GRO expression was largely confined to allogeneic tissue. (Fig 3.33). MCP-1 and MIP-1α expression occurred in mismatched transplants only (Days 1-7). (Fig 3.34, Fig. 3.35) In contrast, MIP-2 mRNA was

detected in all specimens but was not upregulated in acutely rejecting organs. (Fig 3.36).

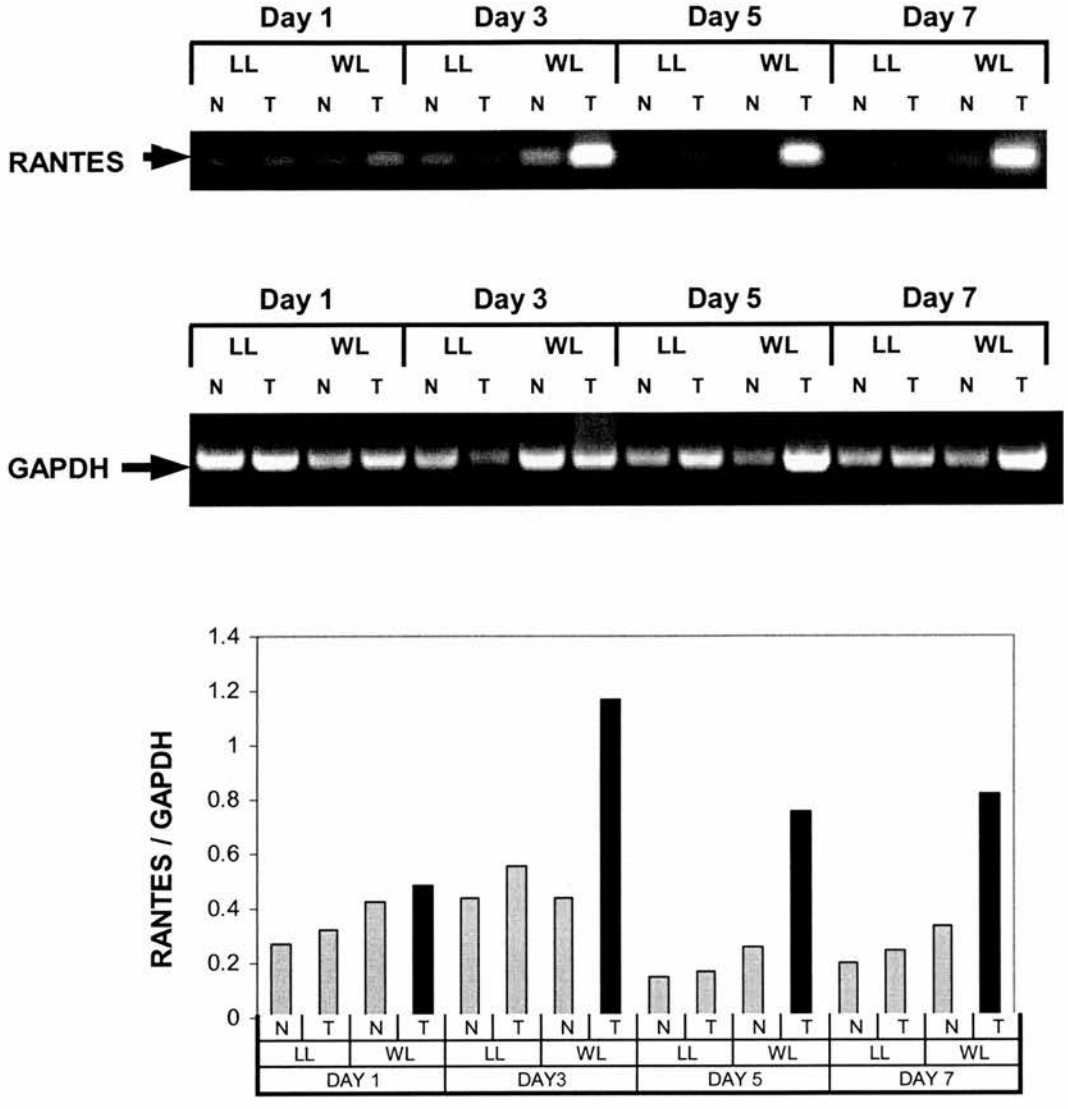


Figure 3.32 RANTES expression in experimental acute cardiac transplant rejection

Expression of RANTES was constitutive in all specimens. mRNA levels for this chemokine were elevated in allogeneic allografts as compared to controls on days 3, 5 and 7

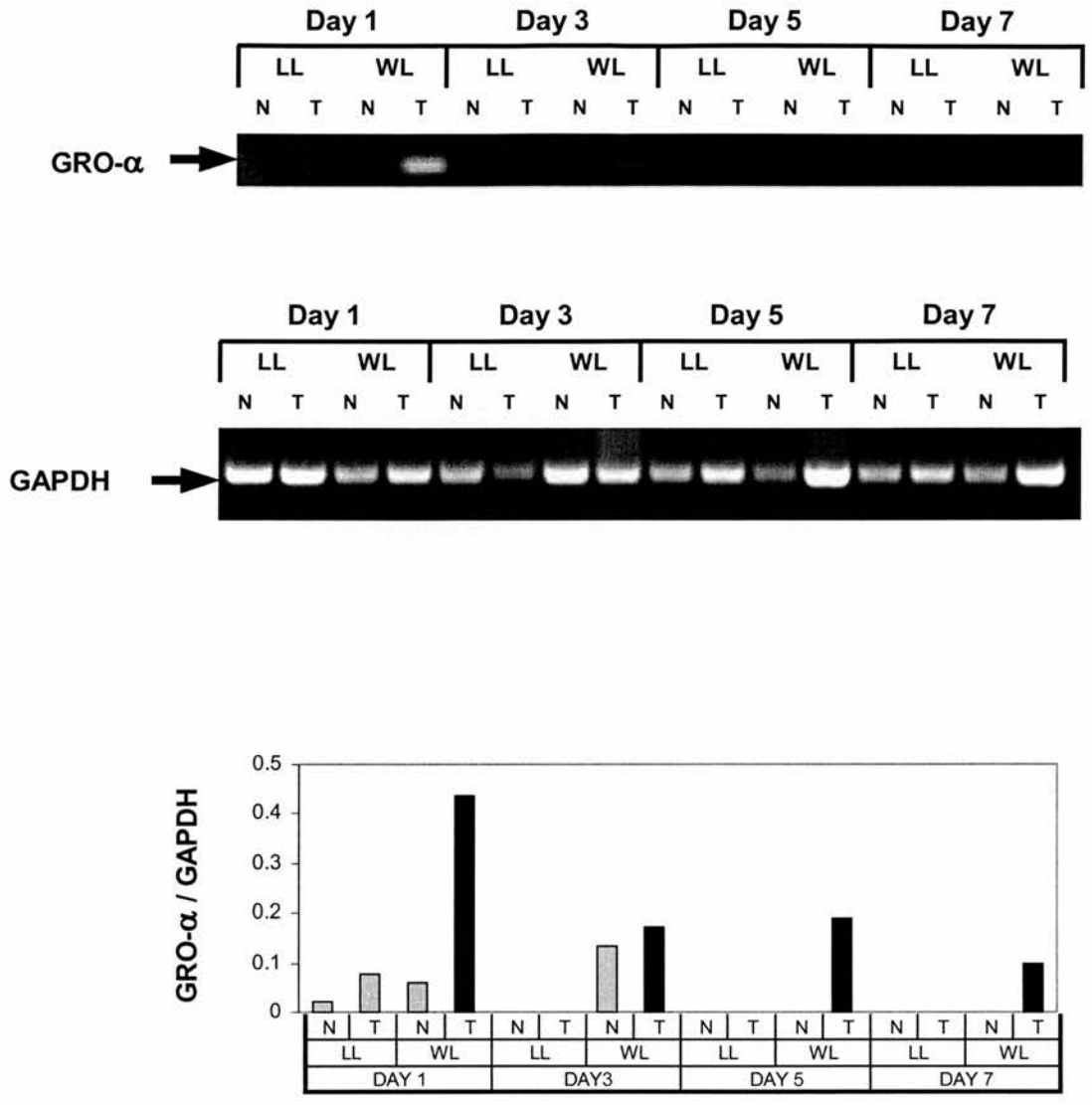


Figure 3.33 GRO-α expression in experimental acute cardiac transplant rejection

.mRNA for GRO-α was detected in control specimens at day 1, while levels in the mismatched transplant at this time-point were markedly elevated. On days 3-7, GRO expression was largely confined to allogeneic tissue.

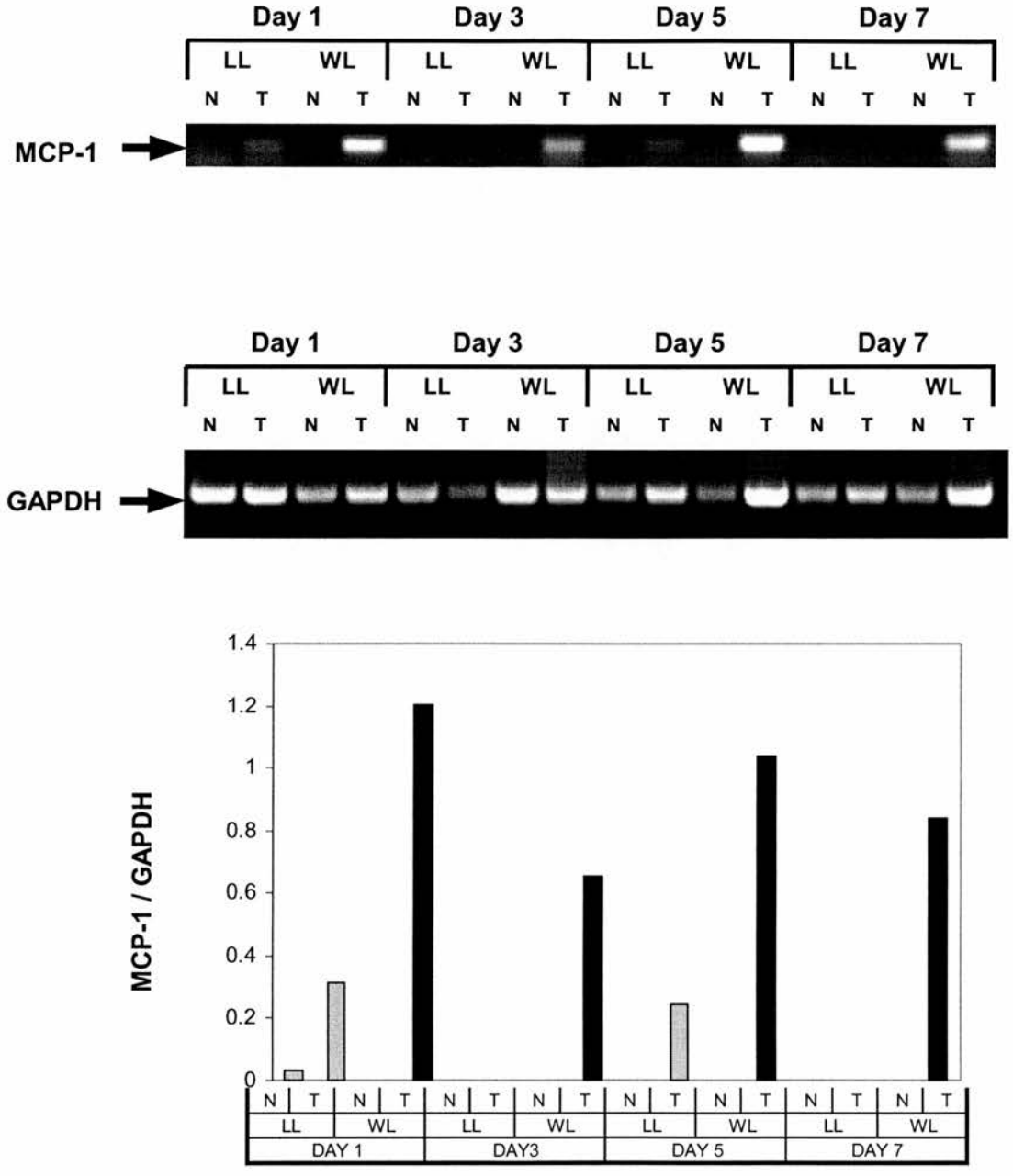


Figure 3.34 MCP-1 expression in experimental acute cardiac transplant rejection

MCP-1 expression was largely confined to mismatched transplants on days 1, 3, 5 and 7.

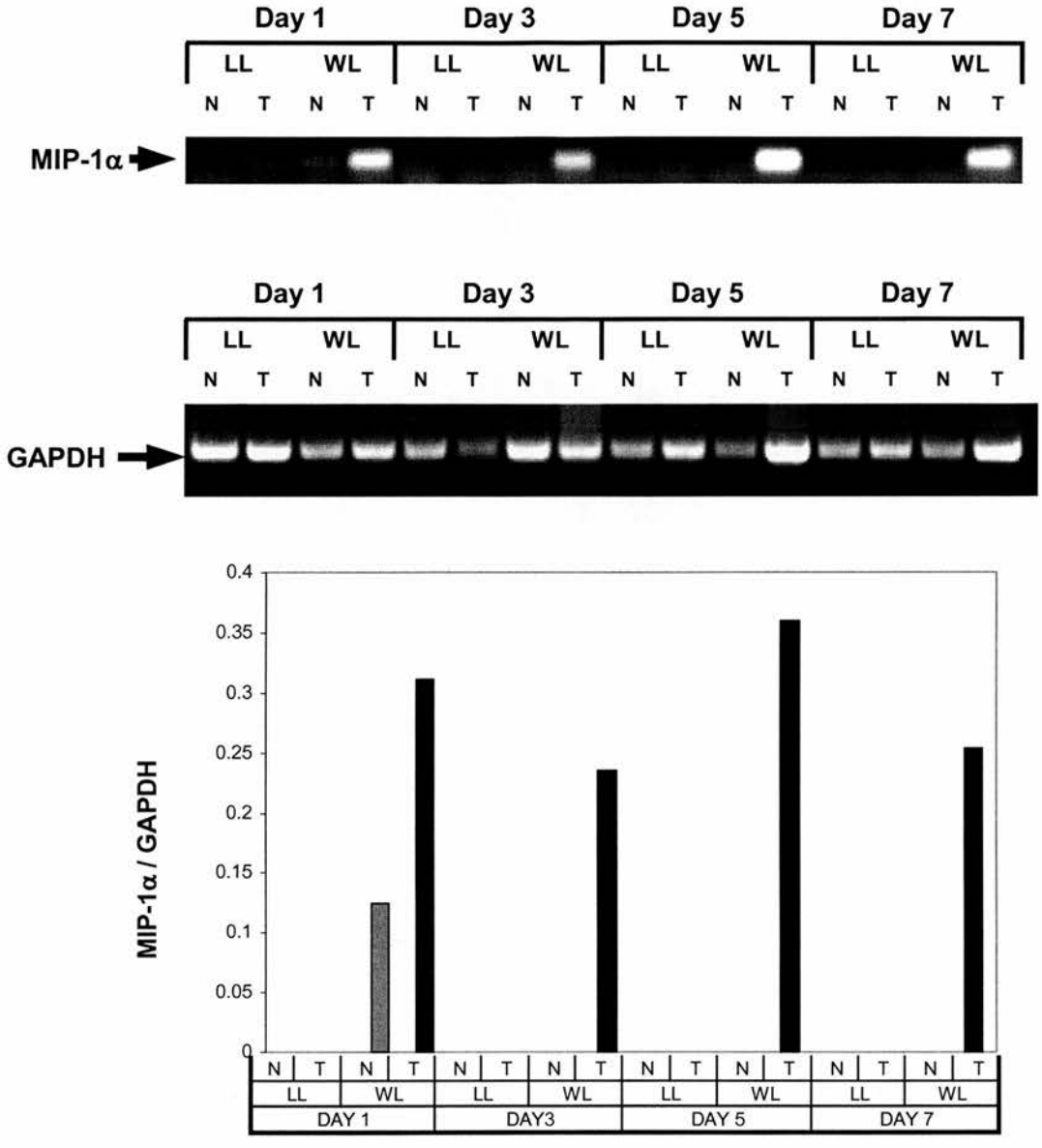


Figure 3.35 MIP-1α expression in experimental acute cardiac transplant rejection

MIP-1α expression occurred in mismatched transplants on days 1, 3, 5 and 7.

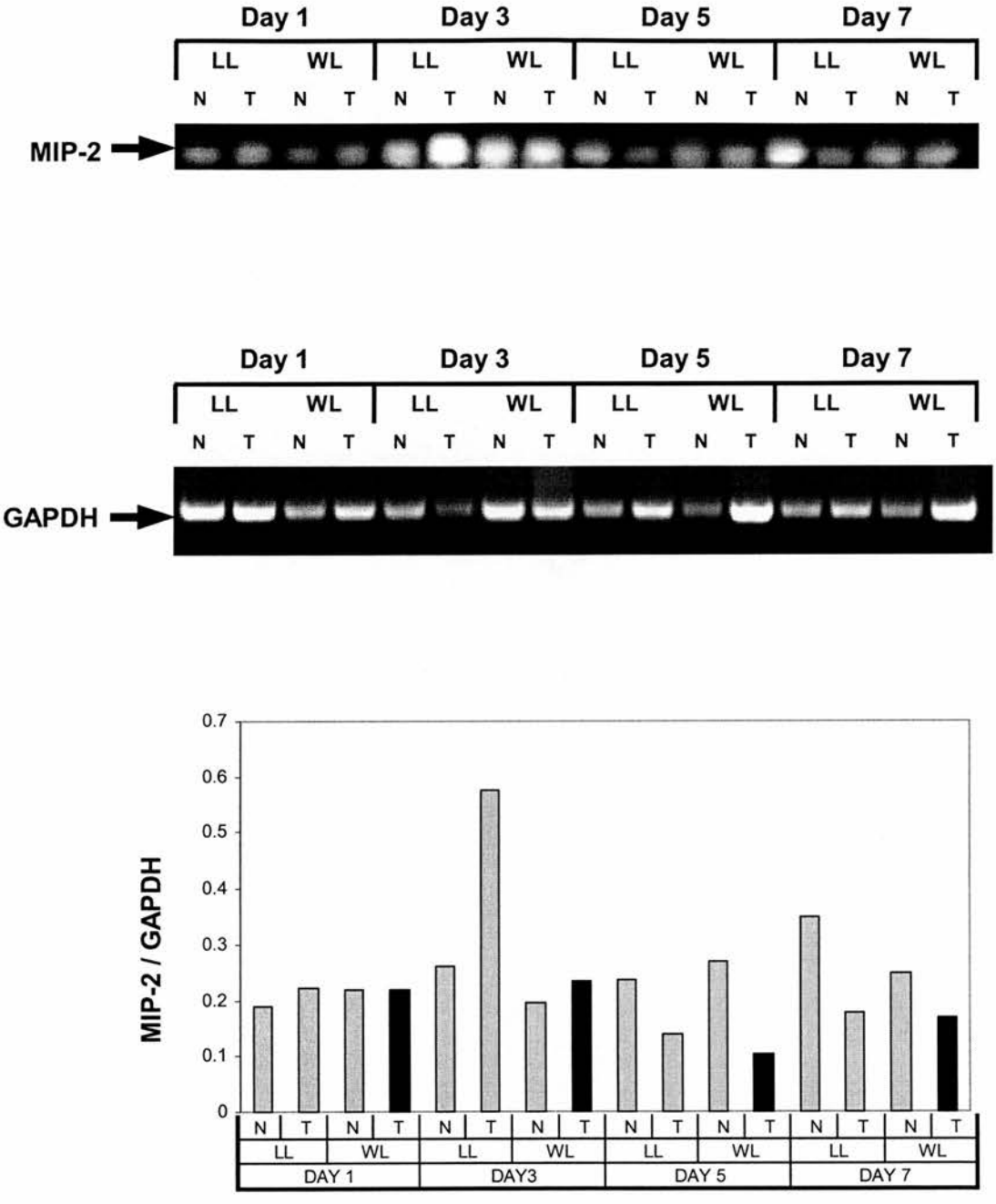


Figure 3.36 MIP-2 expression in experimental acute cardiac transplant rejection

MIP-2 mRNA was detected in all specimens but was not upregulated in acutely rejecting organs

MCP-1 and MIP-1α are monocyte/macrophage chemoattractants, while RANTES is produced by T cells and draws both monocytes and T cells to the target tissue. The C-X-C chemokines GRO-α and MIP-2 are predominantly neutrophil chemoattractants, although GRO has been implicated in atherogenic monocyte/macrophage accumulation. Cells migrating within tissues encounter multiple chemoattractant signals in complex spatial and temporal patterns. There is evidence that certain chemokines exert specificity for overlapping leukocyte subpopulations e.g MCP-1, MIP-1α, RANTES and GRO-α are all, to varying degrees, monocyte chemoattractants. It may be, however, that the chemokines demonstrated to be upregulated in the current study, over varying time courses, participate in different ways to guide leukocyte subsets to their tissue destination and ensure ongoing leukocyte recruitment (as evidenced by ongoing cellular infiltration in the immunohistochemical studies). Some support for this theory comes from a recent *in vitro* study in which it was demonstrated that GRO-α and its receptor CXCR2, but not MCP-1 (or its receptor CCR1), mediated the conversion of monocyte rolling into firm shear-resistant arrest on activated endothelium under conditions of physiological flow. In contrast, MCP-1 and CCR2, but not GRO-α/CXCR2 mediated spreading, shape change and subsequent transendothelial migration (142).

The expression of the chemokine MIP-2 has been implicated in ischaemia-reperfusion injury (143). Although constitutive expression of MIP-2 was detected in the current study, there was no evidence of increased expression in any organs in the early days following surgery. Since MIP-2 is predominantly a neutrophil chemoattractant, and given that these cells do not feature predominantly in the acute

rejection process, it is not surprising that upregulation of this chemokine was not detected in allogeneic organs. Conversely, the observations that T cell and monocyte/macrophage chemoattractants are upregulated in this model concurs with studies implicating these leukocyte subpopulations as the principal immune cell mediators of acute transplant rejection.

Having demonstrated the upregulation of various chemokines in the allogeneic organs, the expression of their corresponding receptors was next investigated. The chemokine receptor CCR1 was detected in all specimens and upregulated in allogeneic organs compared to control on days 1-7. (Fig. 3.37) CCR2 was detected in rejecting hearts only (Fig. 3.38). CXCR2 was detected in all specimens at days 1 and 3, but was consistently elevated above control levels in the mismatched transplants. At days 5 and 7 expression occurred exclusively in allogeneic organs. (Fig 3.39) There was no evidence of expression of the chemokine receptor CXCR4 in any specimens. CXCR4 and its corresponding ligand have been discussed in detail in previous sections. To date only one study has documented CXCR4 expression in human allograft kidneys undergoing severe rejection (144). This was not the case in the model studied here.

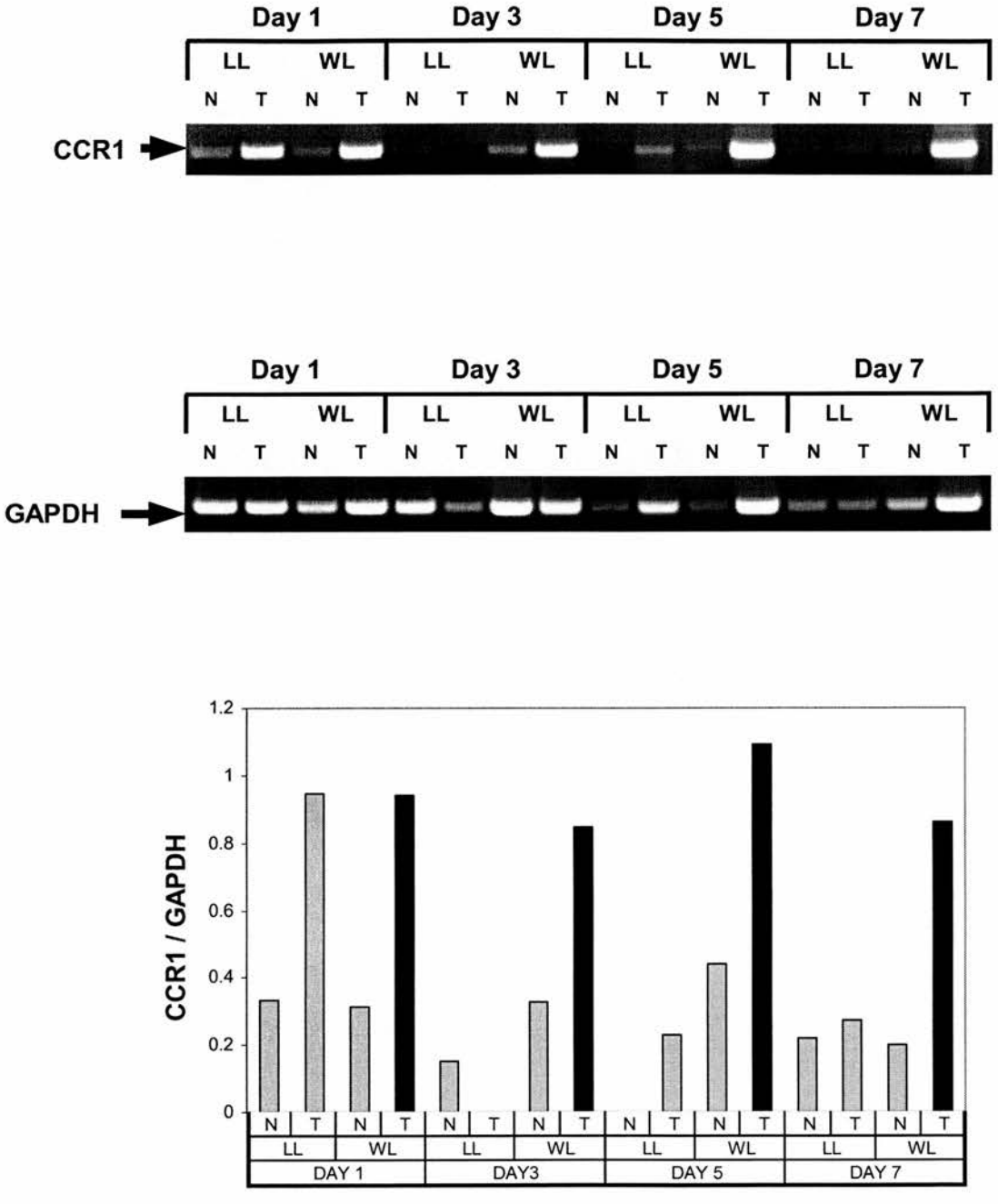


Figure 3.37 CCR1 expression in experimental acute cardiac transplant rejection

CCR1 was detected in all specimens but upregulated in allogeneic organs compared to control on days 1-7. In two samples while PCR fragments were apparent, these proved to be below the sensitivity threshold of the imaging/quantifying equipment.

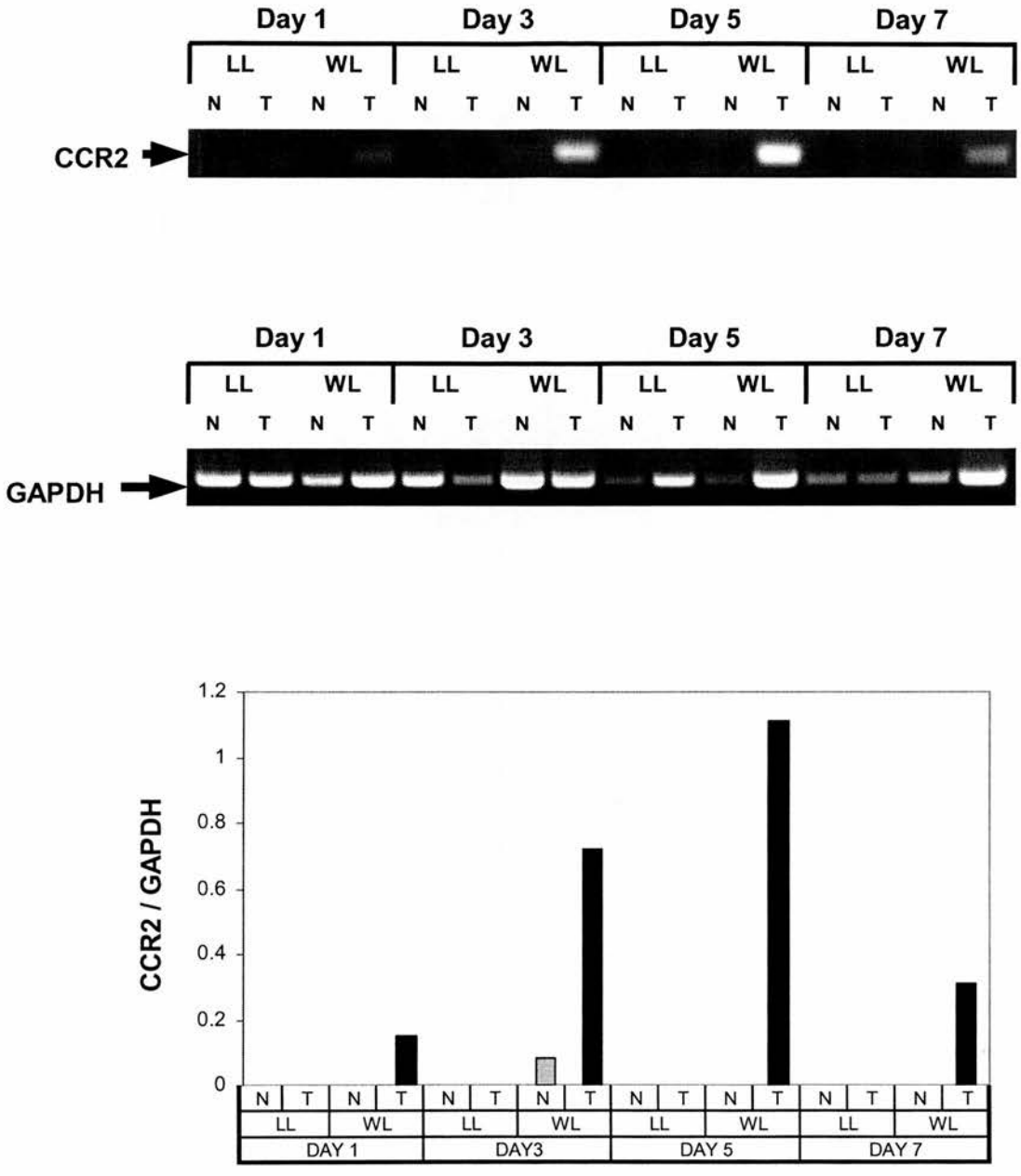


Figure 3.38 CCR2 expression in experimental acute cardiac transplant rejection

CCR2 expression was almost exclusively detected in acutely rejecting mismatched hearts.

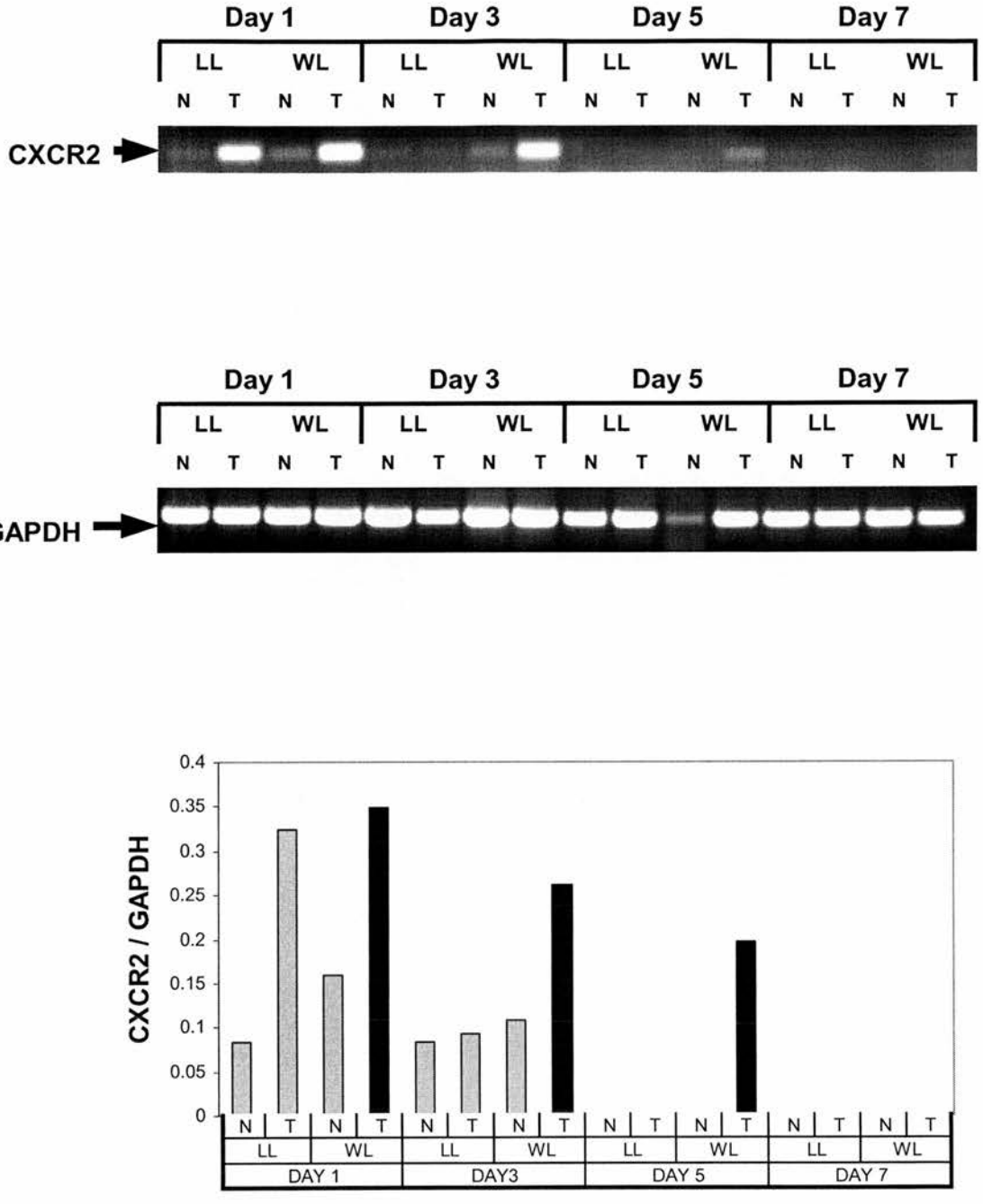


Figure 3.39 CXCR2 expression in experimental acute cardiac transplant rejection

CXCR2 was detected in all specimens at days 1 and 3, but was consistently elevated above control levels in the mismatched transplants. At days 5 and 7 expression occurred exclusively in allogeneic organs. At day 7 a PCR fragment of the expected size was apparent, but this proved to be below the sensitivity threshold of the imaging/quantifying equipment.

Chemokine receptor genes are expressed in a cell-type specific manner and it has been speculated that this may be the basis for the specificity of chemokines for leukocyte subsets. Although predominantly restricted to leukocytes, these receptors have also been demonstrated on other cell types such as VSMC or endothelial cells, either constitutively or in response to inflammatory stimuli. Most chemokine receptors recognise more than one ligand and several chemokines bind to more than one receptor. In relation to the chemokines relevant to the present study, CCR1 binds MIP-1α and RANTES; CCR2 is a receptor for MCP-1 while CXCR2 binds GRO-α and MIP-2. The results of the current study confirm that the upregulation of chemokine expression is paralleled by a concomitant upregulation in cognate receptor expression, for example, increases in MCP-1 mRNA levels occur in tandem with increases in CCR2 mRNA.

Progressive leukocyte infiltration of acutely rejecting hearts has been demonstrated by immunohistology in the current model. Although broadly speaking the expression of the chemokine receptors occur in tandem with increasing immune cell infiltration, it should be noted that chemokine receptor expression is not necessarily restricted to leukocytes as previously discussed. CXCR2 is detected in all organs at days 1 and 3, but after this time expression occurs exclusively in allogeneic organs. This expression pattern suggests that CXCR2 positive cells may be of particular importance in the immune response associated with ischaemia-reperfusion injury.

Little is known about the regulation of chemokine receptor expression *in vivo*. Studies *in vitro* have demonstrated that TNF-α results in downregulation of neutrophil CXC chemokine receptors (145, 146). Increased mRNA levels for TNF-α

in the allogeneic transplants over the time course studied appears to occur in parallel with decreases in CXCR2 expression in these organs. In contrast, interferon-γ and IL-2 have been shown to increase the expression of CCR1 and CCR2 on T lymphocytes and monocytes (147, 148). It is possible that the expression of these cytokines in allogeneic organs at days 5 and 7 may sustain expression of these chemokine receptors. It must be noted that the limitations of semiquantiative RT-PCR prevent a definitive correlation between chemokine receptor expression and cytokine expression.

The importance of the chemokine receptors in leukocyte trafficking and host defence has been illustrated by targeted disruption of these genes in mice. Mice deficient in CCR1 demonstrate impaired host defence, haematopoiesis and granulomatous inflammation (149), while CCR2 deficiency results in impaired monocyte migration and reduced Th1 cytokine responses (150). Binding of a chemokine to its receptor activates the intracellular pathways necessary to propel the cell towards the source of chemoattractant. Activation of the leukocyte also ensues, as evidenced by rises in intracellular calcium, production of microbicidal oxygen radicals and bioactive lipids, and release of lytic enzymes from intracellular granules (Section 1.2.4). These processes are facilitated by each of the many chemoattractant receptors expressed on the leukocyte surface. In the context of allograft rejection, the current model has demonstrated the upregulation of the chemokines RANTES, MCP-1, MIP-1α and GRO-α in allogeneic organs. This is in agreement with the results of other investigators using similar models (143). We have also demonstrated the upregulation of the chemokine receptors CCR1, CCR2 and CXCR2 in the allogeneic organs, and to our knowledge this is the first report of upregulation of these receptors

in this particular model. While we fully accept that the presence of a particular chemokine receptor (or cytokine/chemokine) is not direct evidence of its participation in the rejection response, these novel results raise exciting possibilities. It has become clear, however, from the recent explosive growth in information concerning chemokines and their functions that multiple chemokines can (1) bind to a single chemokine receptor and (2) attract the same leukocyte subpopulations. It may be therefore that immunomodulation at the level of chemokine gene expression could be difficult and possibly ineffective. The discovery that chemokine receptors act as cofactors for HIV infection has boosted the search for therapeutic strategies targeting chemokine receptors and a number of chemokine receptor antagonists now exist which are undergoing preliminary evaluation (151). For example, a truncated form of MCP-1 acts as an antagonist at the CCR2 receptor and prevents the chronic inflammatory arthritis that develops in MRL-lpr mice. In a murine model of acute inflammation, CXCR2 antagonists inhibit neutrophil recruitment. A recent study has demonstrated for the first time that inbred mice with a targeted deletion of CCR1 resulted in a prolongation of cardiac allograft survival exchanged across a MHC class II mismatch. In addition, levels of cyclosporin that had marginal effects in $CCR1^{+/+}$ mice resulted in permanent allograft acceptance in the $CCR^{-/-}$ recipients (152). In light of the results presented here, it would be interesting to examine the influence of chemokine receptor antagonists in the current model both on the temporal nature of allograft rejection and on the expression profile of the immune mediators found to be upregulated in the current study.

Adhesion molecule expression

As has been described previously, while increasing gradients of chemokines attract immune cells to the endothelium, and hence to the target tissue, the successful extravasation of these cells absolutely requires their firm attachment to endothelium via interactions between leukocyte integrins and endothelial expressed VCAM-1 and ICAM-1. Given the crucial role of these adhesion molecules in the paradigm of leukocyte trafficking, we evaluated their expression profile in this model. Although mRNA for VCAM-1 was expressed constitutively, mRNA levels for this leukocyte adhesion molecule and its related molecule ICAM-1 were elevated in rejecting organs as compared to control over the time course studied (Fig. 3.41, Fig. 3.42). Another study of rat cardiac allografts (Dark Agouti donors to WF recipients) has also demonstrated that that ICAM-1 and VCAM-1 expression were markedly upregulated in the acutely rejecting hearts (116). The same study, however, failed to identify VCAM-1 expression on the vascular endothelium in native hearts and demonstrated only weak expression in syngeneic grafts using immunohistochemical techniques. Rat myocytes express VCAM-1 *in vitro* (153) and the observation that VCAM-1 is constitutively expressed in the current study may reflect the fact that whole hearts were used for RNA extraction and that a relatively sensitive detection method i.e. RT-PCR was employed. The crucial role of ICAM-1 in the rejection process has been confirmed in similar experimental models when administration of monoclonal antibodies specific for this adhesion molecule resulted in significant increases in allograft survival (154, 155). Whether such techniques will prove of value in clinical transplantation remains to be seen.

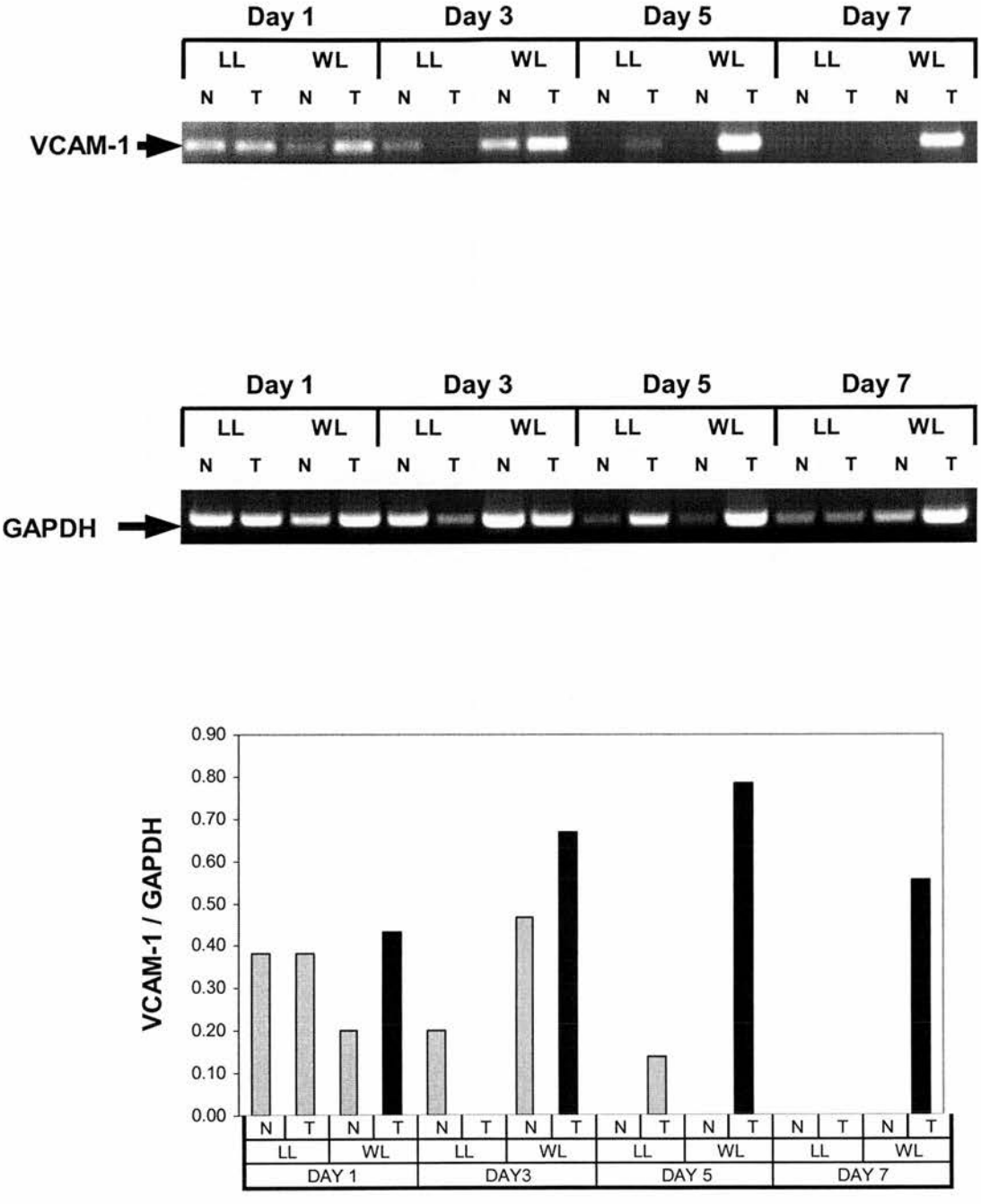


Figure 3.40 VCAM-1 expression in experimental acute cardiac transplant rejection

mRNA for VCAM-1 was expressed constitutively in most specimens but mRNA levels for this leukocyte adhesion molecule were elevated in rejecting organs as compared to controls over the time course studied

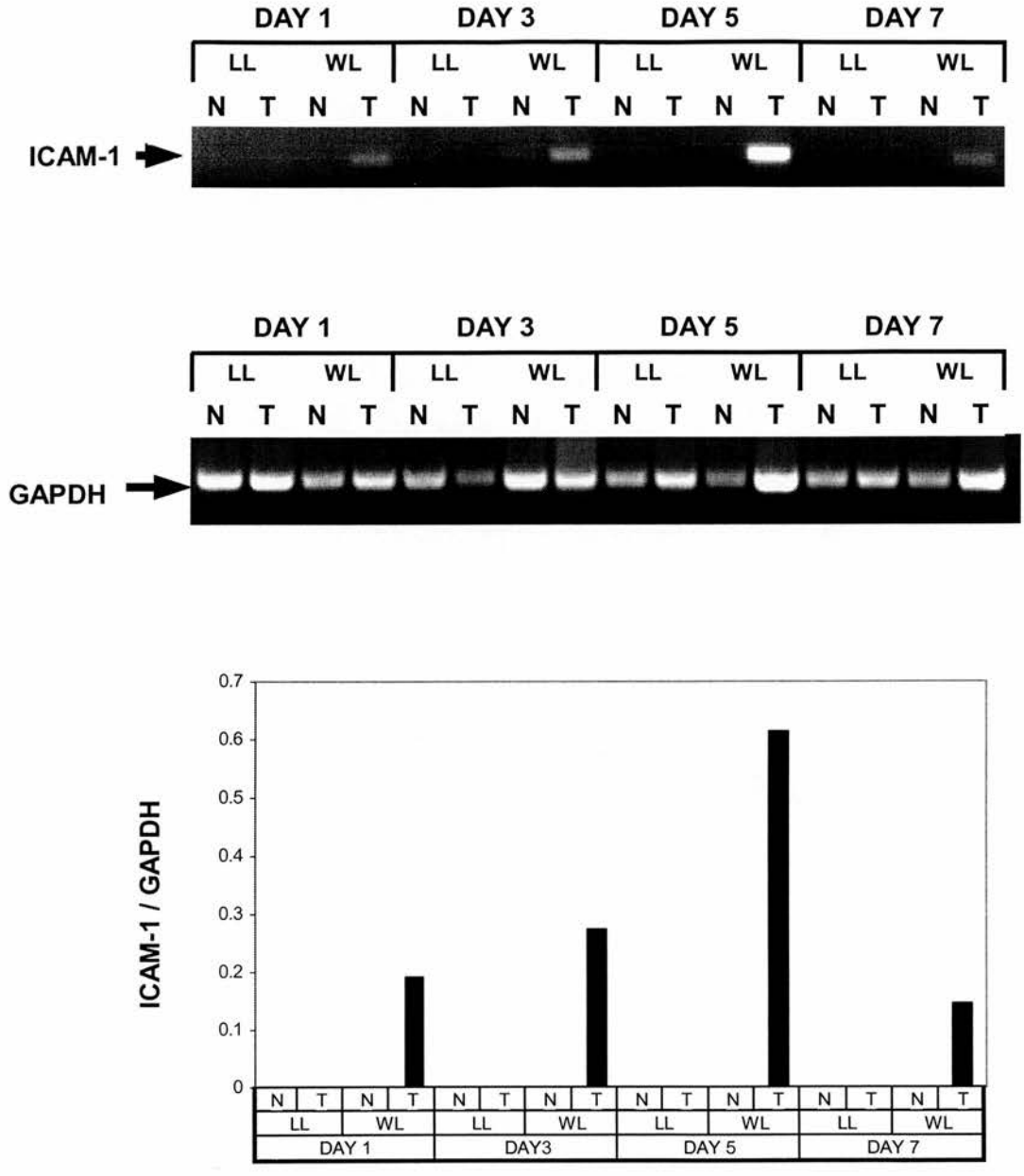


Figure 3.41 ICAM-1 expression in experimental acute cardiac transplant rejection

ICAM-1 expression was detected in mismatched transplants only at days 1, 3, 5 and 7.

Cyclooxygenase II

The role of arachidonic acid metabolites in the inflammatory response is well-established. Arachiodonic acid, released from cellular phospholipase by the actions of phospholipase A_2 is converted to prostaglandins and thromboxane eicosanoids by the actions of cyclooxygenase (COX). This enzyme exists in two isoforms, one present in most cells under basal conditions - COX-1 and one which is rapidly and transiently induced in response to a diverse range of stimuli - COX-2. As discussed in detail in 2.4.3, depending on the inflammatory setting, COX-2 can be potentially deleterious or protective. The role of COX-2 in the allogeneic immune response is currently unknown and on this basis we chose to profile its expression in this model.

mRNA levels for COX-2 were markedly elevated in the mismatched rejecting organs at days 1 and 3 (Fig 3.42). After this time point, expression levels fell markedly with faint and no expression at days 5 and 7 respectively. Weak COX-2 expression was detected in the syngeneic organ at days 1 and 5 only.

The regulation of COX-2 expression is the focus of intense research, most particularly given its pathologic constitutive expression in colon cancer (156). To date, the precise mechanisms underlying its expression *in vivo* have not been elucidated. Broadly speaking, COX-2 expression is regulated by both transcriptional and post-transcriptional mechanisms; the former involves classic transcription factor-dependent promoter activation; the latter, regulation of mRNA translation. Adenosine- and/or uridine-rich – $AUUUA/AU_n$ - elements (AREs) are found in the 3' untranslated (UTR) region of labile mRNA species encoding transiently expressed proteins and have been shown to mediate selective translation-dependent

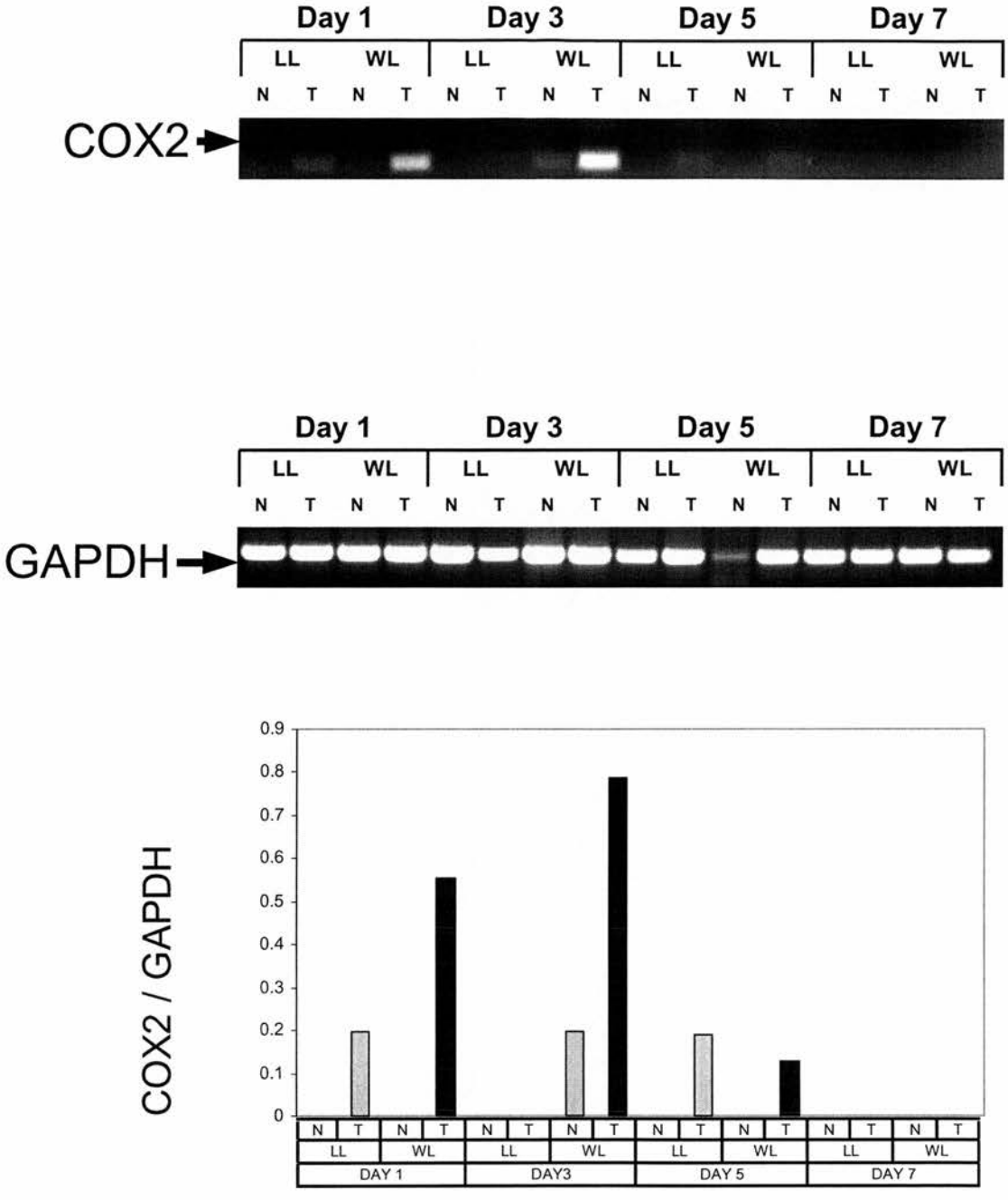


Fig.3.42. mRNA levels for COX-2 were markedly elevated in the mismatched rejecting organs at days 1 and 3. After this time point, expression levels fell markedly with faint and no expression at days 5 and 7 respectively. Weak COX-2 expression was detected in the syngeneic organ at days 1 and 5 only.

destabilization/degradation of such mRNAs (157). A number of ARE motifs are located in the 3'-untranslated region of the human, rat and murine *COX-2* genes; moreover, studies have demonstrated that post-transcriptional regulation of *human* COX-2 expression is mediated by its ARE-rich 3' UTR (158). It is necessary to note, however, that in vitro, cytokine stimulation of various cell types results in *stabilization* of COX-2 mRNA via a p38 MAPK-dependent mechanism (159, 160). Our finding that COX-2 expression was transient and did not persist for the entire time course of the study is, therefore, not wholly unexpected and is probably due to the inherent lability of its mRNA. Moreover, while we have confirmed that increases in the expression of a number of cytokine mRNAs is sustained in the current model, *in vitro* cytokine-stimulation extends COX-2 mRNA half-life from minutes to hours and, therefore, COX-2 expression in the current setting may be viewed, relatively speaking, as prolonged.

Recently another group, using a similar model, have demonstrated that COX-2 expression is upregulated in the rejecting cardiac allografts (161). These authors also demonstrated that increases in COX-2 mRNA levels are paralleled by increases in COX-2 protein and prostaglandin production. Immunostaining revealed increases in COX-2 protein expression in endothelial cells, vascular smooth muscle cells, infiltating macrophages and damaged cardiac myocytes. Interestingly, in animals treated with a selective COX-2 inhibitor, while there was a significant reduction in COX-2 mRNA levels, this was not paralleled by a reduction in myocardial inflammation or rejection grade in allogeneic organs. There was only a slight increase in graft survival from 5.4 to 6.4 days.

It is apparent therefore that COX-2 expression is induced in models of alloantigen-driven inflammation, not altogether surprising given the high levels of infiltrating leukocytes and ambient cytokines in acutely rejecting organs. The exact nature of its role, however, remains unclear. As has previously been discussed, in carrageenin-induced pleurisy in rats, selective COX-2 inhibitors, while inhibiting inflammation at 2 hours, result in a significant exacerbation in inflammation at 48 hours (162). This model at 48 hours is dominated by mononuclear cell recruitment and these authors have suggested that COX-2 expression at this time-point may aid inflammatory resolution by the generation of anti-inflammatory prostaglandins. Likewise the immune response in transplant rejection is dominated by mononuclear cell recruitment and it could be argued that COX-2 production in this scenario is a protective response designed to limit tissue damage. Studies *in vitro* have also demonstrated that COX-2 induction in VSMC in response to cytokines limits their adhesion molecule expression and cytokine release (163). Furthermore in animal blood vessels following injury increases in VSMC COX-2 are associated with increases in PGI_2 and PGE_2 production that compensates for the loss of PG synthesis via constitutive COX-1 in endothelial cells. On this basis it was proposed that COX-2 expression in VSMC may represent a defence mechanism which limits further vessel damage. These observations could potentially explain the lack of response in animals treated with the COX-2 inhibitor.

Alternatively if the induction of COX-2 is viewed as deleterious and responsible for the promotion of tissue damage, it could be argued that the immune response associated with allograft rejection is overwhelming. Often a combination of powerful immunosuppressive agents is needed to ameliorate allograft survival. It might not be

surprising that COX-2 inhibitors alone do not seem to alter this process. However it might be that such treatment in combination with immunosuppressive agents may improve outcomes or indeed reduce the doses of such agents required to suppress rejection. We await with interest further developments in this field.

Summary

The current study examined the expression of SOCS in an experimental model of acute cardiac transplant rejection. In addition, we sought to correlate SOCS expression with immune cell infiltration and the temporal expression of cytokines, chemokines, chemokine receptors and adhesion molecules in this model.

Immunohistology confirmed that, in agreement with the current paradigm of acute allograft rejection, immune cell infiltration of the rejecting hearts was rapid and sustained over the time course studied. This occurred in tandem with increased expression of cytokines (IL-1β, TNF-α, IL-2, IFN-γ, TGF-β and IL-10), chemokines (GRO-α, RANTES, MCP-1, MIP-1α), adhesion molecules (ICAM-1 and VCAM-1) and COX-2 in these organs. Of particular interest is the novel finding that increased chemokine receptor (CCR1, CCR2 and CXCR2) expression occured in allogeneic grafts, suggesting that chemokine receptor antagonists could potentially modify the rejection process in this model.

In addition, allogeneic-specific upregulation of SOCS-3 occurred at all time points. In contrast, increases in CIS-1 and SOCS-1 expression occurred in the mismatched organs at days 3 and 5, respectively. The identify of the particular cytokines (or chemokines) inducing the expression of these SOCS species is as yet unknown. To our knowledge, this is the first report of SOCS expression in experimental cardiac transplant rejection. These findings raise the possibility that these proteins may be important in the modulation of the immune response leading to allograft rejection.

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Future directions.

Exposure of mesangial cells to cyclic mechanical strain results in increases in mRNA for TGF-β and CTGF. It has been demonstrated by other investigators in our group that high glucose stimulates mesangial CTGF expression in vitro by TGF-β dependent and protein kinase C-dependent pathways. The use of anti-TGF-β antibodies will establish whether cyclic mechanical strain induces CTGF through similar mechanisms. Stretch-induced increases in protein surface expression/secretion may occur independently of transcriptional/translational mechanisms; it will be important to investigate whether the surface expression of leukocyte trafficking determinants, such as the chemokine receptors are increased in response to cyclic mechanical strain in this manner.

To our knowledge, this is the first report of SOCS expression in experimental animal models of inflammation. Current work in our laboratory is using dual staining techniques is attempting to define the cell types expressing SOCS in these models. It will be of great interest to define the physiological roles of these proteins in inflammatory diseases since, given their ability to modulate cytokine bioactivity in vitro. In particular, it will be intriguing to explore the possibility that SOCS mimetics could be used as novel anti-inflammatory agents in vivo.

Appendix 1.

Quantification data (raw volumes) for each gene studied in comparison to the GAPDH loading control is detailed below for individual experiments.

Appendix 1A - Influence of mechanical strain on cytokine-adhesion molecule networks in ECV-304 cells

ICAM-1

		C24	S24	C48	S48
EXPT1	ICAM-1	25715.42	214291.77	46156.00	201790.59
	GAPDH	71149.72	58299.69	68286.09	57266.47
	RATIO	0.36	3.68	0.68	3.52

		C24	S24	C48	S48
EXPT2	ICAM-1	23814.36	208876.43	36554.87	236753.95
	GAPDH	89567.34	64509.74	88564.45	76439.65
	RATIO	0.27	3.24	0.41	3.10

		C24	S24	C48	S48
EXPT3	ICAM-1	15743.96	165964.97	21754.39	98765.66
	GAPDH	98677.34	56743.95	76849.49	65976.99
	RATIO	0.16	2.92	0.28	1.50

MCP-1

		C24	S24	C48	S48
EXPT1	MCP-1	153067.78	77536.18	118979.50	640521.52
	GAPDH	71149.72	58299.69	68286.09	57266.47
	RATIO	2.15	1.33	1.74	11.18

		C24	S24	C48	S48
EXPT2	MCP--1	180749.57	94632.12	145389.09	498543.44
	GAPDH	61900.26	44890.76	62823.20	48103.83
	RATIO	2.92	2.11	2.31	10.36

		C24	S24	C48	S48
EXPT 3	MCP-1	232701.47	158721.23	153340.85	577045.84
	GAPDH	95340.62	86283.54	101746.27	86472.37
	RATIO	2.44	1.84	1.51	6.67

IL-8

		C24	S24	C48	S48
EXPT 1	IL-8	98279.00	214449.00	60667.00	101290.00
	GAPDH	76005.50	83697.50	78310.00	86487.50
	RATIO	1.29	2.56	0.77	1.17

		C24	S24	C48	S48
EXPT 2	IL-8	73709.25	145825.32	43680.24	95212.60
	GAPDH	41803.03	51222.87	37588.80	58811.50
	RATIO	1.76	2.85	1.16	1.62

		C24	S24	C48	S48
EXPT 3	IL-8	131693.86	330251.46	107380.59	189412.30
	GAPDH	77197.99	81806.44	79264.03	84249.79
	RATIO	1.71	4.04	1.35	2.25

RANTES

		C24	S24	C48	S48
EXPT 1	RANTES	160404.07	101633.00	121402.00	138645.00
	GAPDH	105558.15	108007.00	91798.17	102977.33
	RATIO	1.52	0.94	1.32	1.35

		C24	S24	C48	S48
EXPT 2	RANTES	135076.27	83875.29	104423.05	130360.39
	GAPDH	132094.41	142582.70	121266.59	139232.01
	RATIO	1.02	0.59	0.86	0.94

		C24	S24	C48	S48
EXPT 3	RANTES	150908.15	90275.41	116886.45	123623.42
	GAPDH	118272.35	132045.84	101804.05	123063.13
	RATIO	1.28	0.68	1.15	1.00

CXCR4

		C24	S24	C48	S48
EXPT 1	CXCR4	84258.49	34847.33	23824.31	41067.50
	GAPDH	71149.72	58299.69	68286.09	57266.47
	RATIO	1.18	0.60	0.35	0.72

		C24	S24	C48	S48
EXPT 2	CXCR4	98579.85	54168.45	31626.30	52486.20
	GAPDH	59052.76	54589.47	73129.29	63172.72
	RATIO	1.67	0.99	0.43	0.83

		C24	S24	C48	S48
EXPT 3	CXCR4	75364.80	22542.15	26579.55	37009.50
	GAPDH	95318.97	81669.11	74115.85	72742.53
	RATIO	0.79	0.28	0.36	0.51

COX-1

		C24	S24	C48	S48
EXPT-1	COX-1	217918.00	140977.50	132892.50	134140.00
	GAPDH	71149.72	58299.69	68286.09	57266.47
	RATIO	3.06	2.42	1.95	2.34

		C24	S24	C48	S48
EXPT-2	COX-1	187652.29	140739.22	130831.18	153336.25
	GAPDH	66190.58	54088.02	72316.58	61049.65
	RATIO	2.84	2.60	1.81	2.51

		C24	S24	C48	S48
EXPT-3	COX-1	206847.77	122060.87	122060.87	152033.11
	GAPDH	60745.92	55734.38	66061.19	52848.95
	RATIO	3.41	2.19	1.85	2.88

COX-2

		C24	S24	C48	S48
EXPT 1	COX-2	124875.00	44512.97	22162.60	49422.00
	GAPDH	71149.72	58299.69	68286.09	57266.47
	RATIO	1.76	0.76	0.32	0.86

		C24	S24	C48	S48
EXPT 2	COX-2	130332.04	51728.79	29559.31	65139.95
	GAPDH	67535.31	54536.26	72615.40	53829.80
	RATIO	1.93	0.95	0.41	1.21

		C24	S24	C48	S48
EXPT 3	COX-2	116047.65	40780.89	30927.79	58844.91
	GAPDH	68621.56	55964.69	63741.80	60386.97
	RATIO	1.69	0.73	0.49	0.97

Appendix 1B - Influence of cyclic mechanical strain on glomerular endothelial and mesangial cell activation *in vitro*

TGF-β

EXPT 1

	C24	S24	C48	S48	C72	S72
TGF	39129.40	58606.00	53572.00	51270.00	63717.20	67160.40
GAPDH	68158.33	77431.86	71314.69	33534.10	80867.00	32562.45
RATIO	0.57	0.76	0.75	1.53	0.79	2.06

EXPT 2

	C24	S24	C48	S48	C72	S72
TGF	37913.66	62685.94	55133.42	44710.93	71928.79	62197.25
GAPDH	74706.45	81627.00	66366.30	43475.25	82514.25	40281.15
RATIO	0.51	0.77	0.83	1.03	0.87	1.54

EXPT 3

	C24	S24	C48	S48	C72	S72
TGF	45768.29	65102.75	60722.28	53622.91	62232.79	64045.39
GAPDH	63527.10	67785.90	62994.75	37087.05	76126.05	26794.95
RATIO	0.72	0.96	0.96	1.45	0.82	2.39

CTGF

EXPT 1

	C24	S24	C48	S48	C72	S72
CTGF	244920.04	220133.00	121290.96	267014.98	131979.24	233941.77
GAPDH	68158.33	77431.86	71314.69	33534.10	80867.00	32562.45
RATIO	3.59	2.84	1.70	7.96	1.63	7.18

EXPT 2

	C24	S24	C48	S48	C72	S72
CTGF	232142.82	197938.20	142425.79	239432.33	163172.85	266908.17
GAPDH	77098.60	86608.56	76024.99	43286.96	91302.74	41414.92
RATIO	3.01	2.29	1.87	5.53	1.79	6.44

EXPT 3

	C24	S24	C48	S48	C72	S72
CTGF	329149.37	272338.19	185245.26	288927.31	127183.31	277176.68
GAPDH	64039.74	67682.72	64039.74	28185.16	74585.21	25884.33
RATIO	5.14	4.02	2.89	10.25	1.71	10.71

IL-8

EXPT 1

	C24	S24	C48	S48	C72	S72
IL-8	197735.00	124588.93	185158.56	34798.40	209078.33	73014.00
GAPDH	68158.33	77431.86	71314.69	33534.10	80867.00	32562.45
RATIO	2.90	1.61	2.60	1.04	2.59	2.24

EXPT 2

	C24	S24	C48	S48	C72	S72
IL-8	126011.51	72006.58	131280.28	18879.77	146647.54	43028.32
GAPDH	58927.78	68437.74	63343.12	31416.83	73532.36	24454.18
RATIO	2.14	1.05	2.07	0.60	1.99	1.76

EXPT 3

	C24	S24	C48	S48	C72	S72
IL-8	171235.15	116791.16	162453.86	34798.40	193188.38	78153.48
GAPDH	70985.05	79306.27	77345.09	36563.89	90684.25	39039.57
RATIO	2.41	1.47	2.10	0.95	2.13	2.00

Appendix 1C - Regulation of SOCS expression in glomerular endothelial and mesangial cells *in vitro*

SOCS-3 (glomerular endothelial cells).

EXPT 1

	Control	IL-1	IL-4	IL-6	TNF	IFN
SOCS-3	50272.00	107759.00	95750.00	109201.00	113574.00	156926.00
GAPDH	92783.00	95524.00	97147.00	97951.00	99987.00	96405.00
RATIO	0.54	1.13	0.99	1.11	1.14	1.63

EXPT 2

	Control	IL-1	IL-4	IL-6	TNF	IFN
SOCS-3	59782.99	94249.76	80738.43	95238.39	107761.08	170253.12
GAPDH	94430.00	100440.00	101240.00	100800.00	104570.00	99310.00
RATIO	0.63	0.94	0.80	0.94	1.03	1.71

EXPT 3

	Control	IL-1	IL-4	IL-6	TNF	IFN
SOCS-3	77556.32	119058.01	87654.80	124000.00	133703.86	159555.96
GAPDH	92110.00	93200.00	99410.00	103340.00	98400.00	95280.00
RATIO	0.84	1.28	0.88	1.20	1.36	1.67

CIS-1 (glomerular endothelial cells).

EXPT 1

	Control	IL-1	IL-4	IL-6	TNF	IFN
CIS-1	14272.13	27431.86	34322.50	26575.50	45172.50	80392.50
GAPDH	92783.00	95524.00	97147.00	97951.00	99987.00	96405.00
RATIO	0.15	0.29	0.35	0.27	0.45	0.83

EXPT 2

	Control	IL-1	IL-4	IL-6	TNF	IFN
CIS-1	21609.50	37816.63	35621.92	31063.66	54699.06	98762.19
GAPDH	96650.00	102650.00	107936.75	107888.99	112427.59	127728.98
RATIO	0.22	0.37	0.33	0.29	0.49	0.77

EXPT 3

	Control	IL-1	IL-4	IL-6	TNF	IFN
CIS-1	10992.23	21569.66	36754.56	36502.50	47079.93	73627.21
GAPDH	54182.63	64643.64	70276.49	78591.64	94149.03	110242.89
RATIO	0.20	0.33	0.52	0.46	0.50	0.67

SOCS-3 (mesangial cells)

EXPT 1

	Control	IL-1	IL-4	IL-6	TNF	IFN
SOCS 3	231220.20	247985.40	134606.00	131617.40	161477.20	307627.00
GAPDH	80538.50	78161.00	77489.00	74323.50	57933.50	87024.50
RATIO	2.87	3.17	1.74	1.77	2.79	3.53

EXPT 2

	Control	IL-1	IL-4	IL-6	TNF	IFN
SOCS 3	244840.33	286831.41	167964.34	175716.54	211247.46	416839.69
GAPDH	101853.93	98390.89	99032.57	66948.59	65879.12	97963.11
RATIO	2.40	2.92	1.70	2.62	3.21	4.26

EXPT 3

	Control	IL-1	IL-4	IL-6	TNF	IFN
SOCS 3	190829.21	201333.57	122550.87	103292.88	182075.58	281867.00
GAPDH	75290.42	62029.04	85129.51	81279.43	69729.20	80637.75
RATIO	2.53	3.25	1.44	1.27	2.61	3.50

CIS-1 (mesangial cells)

EXPT 1

	Control	IL-1	IL-4	IL-6	TNF	IFN
CIS-1	29834.69	35250.19	30201.85	22275.12	40108.77	38442.42
GAPDH	80538.50	78161.00	77489.00	74323.50	57933.50	87024.50
RATIO	0.37	0.45	0.39	0.30	0.69	0.44

EXPT 2

	Control	IL-1	IL-4	IL-6	TNF	IFN
CIS-1	46157.17	53408.46	46769.79	41161.90	51753.11	60687.62
GAPDH	67800.79	60400.00	74928.09	67983.54	47698.13	79131.38
RATIO	0.68	0.88	0.62	0.61	1.09	0.77

EXPT 3

	Control	IL-1	IL-4	IL-6	TNF	IFN
CIS-1	39578.88	37773.53	36801.42	31524.23	54577.20	46800.29
GAPDH	72004.07	73283.33	73831.59	72186.82	50987.65	76755.61
RATIO	0.55	0.52	0.50	0.44	1.07	0.61